An insight into the role of 9360FtsH protease in photoprotection in the cyanobacterium *Synechococcus* PCC7942

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by

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ABSTRACT

The multisubunit complex of photosystem-II, present in thylakoid membranes of all oxygen evolving photosynthetic organisms has some unique features. It drives one of the most thermodynamically demanding reactions, the oxidation of water. Yet it turns over more rapidly than any other protein complex of the photosynthetic apparatus and its D1 core protein, binding the majority of electron transport cofactors, is the most frequently damaged subunit of all. The repair mechanism operating to restore photosystem II to its functional state requires proteolytic activity to degrade the photo-inactivated D1 subunit before replacing it with a newly synthesized copy.

In our model organism, the cyanobacterium *Synechococcus* 7942, we explored the possibility that an FtsH protease (JGI ID 637799360) plays an important role in the early stages of the repair cycle by subjecting the gene encoding it to insertional mutagenesis. The phenotype of the resulted mutant was subsequently compared to that of the wild type, as both types of cells were simultaneously assessed using various biochemical and biophysical techniques.

The assay produced results correlating well with the present knowledge about the role of the particular protease in other model organisms such as *Synechocystis* 6803 and *Arabidopsis thaliana* and thus substantiated the significance of the protease in the repair cycle of photosystem II and yet proposed an evolutionary conserved role among oxygenic phototrophs. We identified a possible role in the degradation of functional photosystem II under stress conditions and its dynamics within thylakoid membranes since absence of this protease profoundly affects the diffusion of the complex in the membranes. Besides, computational analysis of FtsH proteins, present as multigene families in all oxygenic phototrophs, brought forth the discovery of a unique domain present in cyanobacterial peptidases.

Dedicated to my daughter Maria-Aelia

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ABBREVIATIONS

AGE - Agarose Gel Electrophoresis

APC - Allophycocyanin

BL - Blue Light

BLAST - Basic Local Alignment Search Tool

BLOSUM - Blocks Substitution Matrix

CAB proteins - Chlorophyll a / b binding proteins

CIAP - Calf Intestinal Alkaline Phosphatase

CM - Cytoplasmic membrane

da - dark adapted

ETS - Electron Transport System

FNR - Ferrodoxin-NADP-oxireductase.

FRAP - Fluorescence Recovery After Photobleaching

Fts - Filament-forming Temperature Sensitive

GL - Green Light

GOLD - Genome On Line Database

IMG - Integrated Microbial Genomes

JGI - Joint Genome Institute

LB - Luria Bertani broth.

LH - Light Harvesting

LHC - Light Harvesting Complex

LPS - Lipopolysaccharides

LSCM - Laser Scanning Confocal Microscopy

MSA - Multiple Sequence Alignment

NCBI - National Centre for Biotechnology Information

NPQ - Non-Photochemical Quenching

OCP - Orange-Carotenoid Protein

OD - Optical Density

ORF - Open Reading Frame

PB - phycobilins

PBP - phycobiliproteins

PBS - phycobilisomes

PC - Phycocyanin

PCC - Pasteur Culture Collection

PE - Phycoerethrin

PEC - Phycoerythrocyanin

Pftf - Plastid fusion/protein translocation factor;

PS - photosystem

PS-I - photosystem I

PS-II - photosystem II

ra - red (light) adapted

RC - Reaction Centre

RL - Red Light

ROS - Reactive Oxygen Species

RT - Room Temperature

SCP - Small CAB proteins.

Tat - Twin Arginine Transport

Taxid - Taxonomy ID

TEM - Transmission Electron Microscopy

TM - Thylakoid Membranes

TP - Targeting Peptide

WOC - Water Oxidation Complex

CHAPTER I

INTRODUCTION

Chapter I

Introduction

1. Photosynthesis

Photosynthesis, which literally means synthesis with light, is a biological process by which an organism captures the electromagnetic energy of sunlight and converts it into biochemical free energy that drives the cellular processes. Seemingly simple as concept, and with fairly straightforward overall equation, photosynthesis is a very complex physiological phenomenon that comprises a series of very diverse and highly coordinated events, ranging from pure physical processes as the capture of light and energy transfer, to sheer enzymatic reactions as of carbon assimilation.

However, referring to photosynthesis by reflecting merely the conversion of electromagnetic energy of sunlight into chemical energy in organic molecules, and taking no notice of its extraordinary intrinsic ability to sense the environmental changes and respond to them by balancing the energy input through biophysical, and photochemical reactions with the energy output consumed by the metabolic sink itself, would simply hide a large part of the beauty and greatness of this process.

The sunlight and the process of photosynthesis are essential elements for the sustenance of life on earth, in the form that it exists today.

Energy is the requisite for life, or in other words, all living organisms need to find a way to obtain energy to sustain life. Throughout their evolutional history, organisms have developed different mechanisms for this purpose. Some have evolved an apparatus that captures the electromagnetic energy of sunlight, hence called phototrophic, while others gain the required energy from oxidizing organic or / and inorganic matter and therefore referred to as chemotrophic. This classification based on metabolic patterns, though does not reflect any evolutionary or taxonomical relationships, is very useful for it laconically reveals the patterns of energy flow in the described species. However, as the degree to which an organism utilizes one or both patterns to gain energy varies amongst different species as well as between individuals of the same species depending on the environmental conditions, there is no clear cut-line between these two distinct metabolic life forms, namely phototrophic and chemotrophic.

Photosynthesis while furnishing the organisms capable of performing it with energetic independence, provides ultimately the source of metabolic energy for the vast majority of species on earth. William Ralph Inge, a prominent English author, and an Anglican priest of 19th century, commenting on life very aptly noted: " The whole of nature, as has been said, is a conjugation of the verb to eat, in the active and passive"

The emergence of photosynthesis and its evolution to the oxygen evolving form resulted not only in the oxygen rich environment that still exists today, but has fundamentally changed the face of the earth. Accumulation of oxygen in the atmosphere led to formation of ozone layer that absorbed, as it still does, a notable amount of ultraviolet (UV) radiation, allowing thus the cells to colonize the surface waters and eventually the land. Without the ozone shield in the upper part of the atmosphere the UV radiation would have caused unsustainable levels of mutations in exposed cells. Moreover, oxygen, although a dangerous reactive species, became for countless organisms, including us humans, the means to obtain energy from organic matter.

After all, the best way to appreciate the importance of photosynthesis may be not the analysis of its impact on the life on this planet but the examination of the consequences of its absence...

The word photosynthesis as well as any of its derivatives, e.g. photosynthetic, almost instinctively relates to green color. Although this association with the green color is historically justified for our first clues upon this process came from the higher plants that are all around us, and the green being the dominant color, it is worth mentioning that photosynthetic organisms are not only versatile in their shape, size, cell structure and organization but in color as well for many of them are not green at all as for instance purple bacteria, brown and red algae and numerous higher plants, to name just the cherry plum, *Codiaeum* sp and numerous others.

Another matter that merits some attention is which organisms are to be called photosynthetic. Historically, this term was first applied to green plants that all shared a common feature. To capture light and convert it into biochemical energy they were invariably using chlorophyll pigments. The advent however of microscopy and the advance in sciences and technology led to the discovery of thousands of bacterial or other eukaryotic unicellular species many of which could also harness the solar energy.

Adopting a broad definition of photosynthesis, as the ability to capture the energy of sun and utilize it in any cellular processes, will inevitably embrace Halobacteria from the domain of Archaea in line with the green plants, algae and photosynthetic bacteria.

Halobacteria are heterotrophic halophiles, which inhabit environments characterized by the presence of high amounts of salt (saturated or nearly so), moisture, and organic matter. These organisms use bacteriorhodopsin and halorhodopsin, as their main pigments, which are similar to the sensory rhodopsin used by humans and other animals for vision. In their energy transducing mechanism there are no light-driven electron transfer processes but only light-induced *cis-trans* isomerizations of the membrane embedded rhodopsin, directly coupled to ion (H^+ or Cl^-) pumping across the membrane (Haupts *et al.,* 1999). They do not use light to generate reducing power since as truly heterotrophs they cannot use CO_2 as their carbon source. The extra energy they obtain from light, is mostly used to maintain their ionic composition.

Unlike Halobacteria, the rest of organisms capable of harnessing the solar energy to any degree, namely green plants, algae and photosynthetic bacteria, all share some common characteristics as far as the photosynthetic apparatus is concerned. Regardless of how different in shape, size, life style, cell structure and organization these organisms may be, ranging from unicellular bacterial species to the giant sequoia (*Sequoiadendron giganteum*) reaching up to 115 meters in hight, all of them use tetrapyrrole ring pigments for light harvesting and perform light-driven electron transport across the membrane (reviewed in Blankenship 2002).

As this chlorophyll-based energy transducing mechanism of eubacterial and eukaryotic organisms radically differs from that of Halobacteria, the latter is not considered by some biologists to be truly photosynthetic.

Regardless however of what one's views upon this matter may be, merely for clarity and consistency, any reference to photosynthesis henceforth will imply the use of tetrapyrrole ring pigments coupled with electron transfer reactions across the membrane, excluding therefore Halobacteria.

2. Classification of photosynthetic organisms

Although there are many ways to classify life, none of the methods is inherently superior to any other, as all such systematic classifications of life are ultimately only for our benefit. However, the bewildering diversity of living organisms almost dictates the necessity to classify and arrange them into groups based on their mutual similarities.

Biological systematics is the study of diversity and evolutionary relationships among living organisms, both extinct and extant, on the planet earth. The term systematics is sometimes used synonymously with 'taxonomy'. However, taxonomy is describing, identifying, classifying and naming the organisms while systematics is focused on placing organisms within groups that show their relationship to other organisms. Systematics uses taxonomy as the main tool in understanding organisms, since the relationship of an organism with other living things cannot be understood without first being properly studied and described in details to identify and classify it correctly.

The emergence of molecular sequencing in combination with the eruption of computational sciences not only have transformed Darwin's vision into a reality, but moved the evolution to molecular level. The central dogma of the emerged new discipline – Molecular evolution, is that the genealogical history of an organism resides to one extent or another in the sequence of its genes. Thus, comparing the sequences of molecules whose distribution is broad or universal, can provide great insight into the evolutionary relatedness among various organisms. Furthermore, identifying and understanding the homologies (shared common ancestor) between proteins involved in the same type of reactions is extremely beneficial and important when considering the next: there are estimated to be around 500,000 species of plants and millions of bacteria and if all those species had evolved diverse mechanisms for photosynthesis, research aiming at understanding the mechanism of photosynthesis would be most definitely absurd and hopeless.

Carl Woese, reliant on the sequence of ribosomal RNA (rRNA) has formulated the three-domain theory of life, namely Eubacteria, Archaea, and Eukarya (Woese 1987; 2000; 2004), Figure I.1.

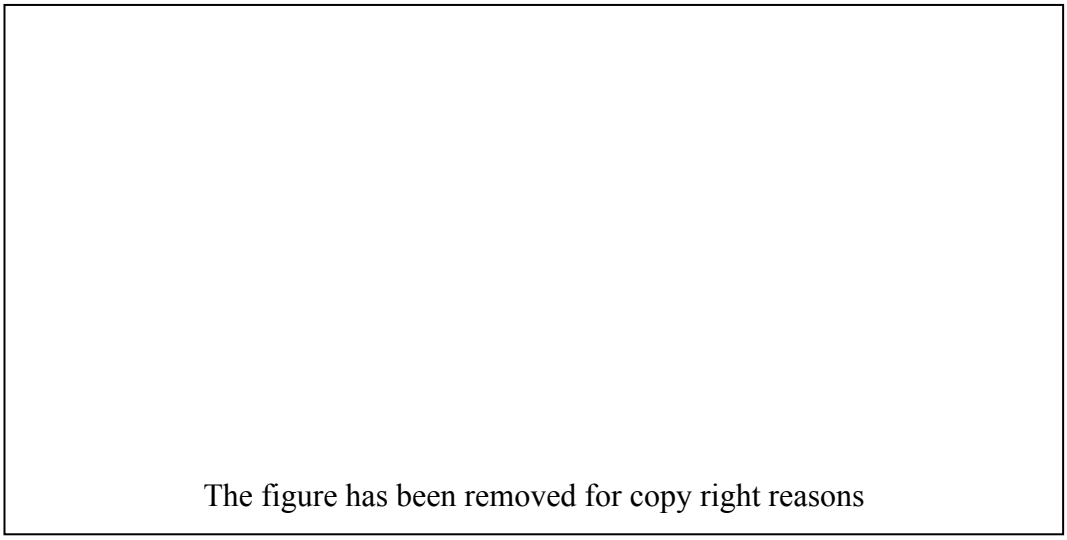


Figure I.1. **The universal phylogenetic tree as determined from the 16S rRNA comparative sequence analyses** (Woese, 2000).

This particular selection of rRNA as molecular chronometers is based on the fact that these molecules are ancient and distributed over all lineages of life and perform the same function in all organisms, with no or very little lateral gene transfer.

Photosynthetic organisms constitute a very large and diverse group that contains species from the domains of Eubacteria and Eukarya. No photosynthetic organisms have been identified thus far in Archaea.

Despite their remarkable diversity, photosynthetic organisms can be classified into two major groups: oxygenic and anoxygenic. In all plants and algae, as well as in certain types of bacteria, the oxygenic photosynthesis, apart from reduction of CO_2 to carbohydrates, also involves removal of electrons from water, resulting in the release of molecular oxygen as a byproduct of the process. Of course, there is no clear-cut line between these two groups, as some cyanobacteria, under certain circumstances, can change their electron donor substrate, switching from H_2O to H_2S for instance (Padan, 1979), and as the result the molecular sulfur to be released as a waste product.

Phylogenetic analysis of the Mg-tetrapyrrole biosynthesis genes indicates that the most anoxygenic photosynthetic organisms are the progenitors of the oxygen evolving cyanobacteria. The lineage of *Proteobacteria* (Purple bacteria), seems to contain the

most ancestral form of this pigment biosynthesis pathway (Xiang *et al.,* 1998; Xiong 2002).

All photosynthetic bacteria are typical aquatic microorganisms inhabiting marine and fresh water environment like moist and muddy soil, stagnant ponds and lakes, sulfur springs etc. Until recently, chlorophyll-based photosynthesis was known to occur within five eubacterial phyla, namely: (i) *Firmicutes*, (ii) *Proteobacteia*, (iii) *Chlorobi*, (iv) *Chloroflexi* and (v) *Cyanobacteria*. With the discovery of *Candidatus chloracidobacterium thermophilum (Chloracidobacterium thermophilum),* just in 2007, Yellowstone USA (Bryant *et al.* 2007), the number of eubacterial phyla containing photosynthetic organisms is now increased to six, the last one (vi) being the phylum of *Acidobacteria*.

Firmicutes

Heliobacteria is the only class in this phylum capable to perform photosynthesis. Considered often to contain the most ancestral form of photosynthesis, Heliobacteia are the only photosynthetic bacteria that stain Gram positively.

Proteobacteria

Proteobacteria, and in particular alpha, beta and gamma Proteobacteria are the only classes in this phylum to contain photosynthetic species, widely known as Purple bacteria.

Chloroflexi and *Chlorobi*,

Commonly known as Green Non-Sulfur and Green Sulfur bacteria respectively, they are the other two phyla with photosynthetic species and with the former to be considered by many as the earliest branching photosynthetic bacteria.

Cyanobacteria

The fifth eubacterial phylum where photosynthesis was known to occur, is the only one that its organisms contain two reaction centres in contrast to the rest of photosynthetic bacteria that either have Type-I or Type-II reaction centres. Although the vast majority of oxygenic photosynthetic bacteria belong to cyanobacteria, there are a few types of O_2 evolving phototrophs, containing chlorophyll *b* and *d*, that are sufficiently distinct from cyanobacteria and thus can be singled out. Phylogenetic analysis, based on 16S rRNA, has shown that oxygenic photosynthetic bacteria form a large, single phylum (Turner.,

1997). Because the phylum is vastly dominated by authentic cyanobacteria, for simplicity sake, all oxygenic photosynthetic bacteria are called cyanobacteria.

3. Cyanobacteria as model organisms

Cyanobacteria, formerly known as blue-green algae, are one of the largest and most important groups of bacteria on earth. Evolved 2.8 to 3.5 billions years ago (Olson 2006; Allwood *et al.,* 2009), they have had a tremendous impact in shaping the course of evolution and ecological change throughout earth's history. The oxygen atmosphere that we all depend on, was generated by cyanobacteria during the Archaean and Proterozoic Eras. Before that time, the atmosphere had a radically different composition and chemistry, unsuitable for life, as we know it today. Almost undoubtedly, they are believed to be the progenitors of the chloroplasts in eukaryotic organisms. Versatile in metabolic lifestyle, heterocyst-forming species are specialised in nitrogen fixation, playing thus an essential role in global agricultural production, in particular that of rice and beans (Roger & Kulasooriya 1980; Bocchi & Malgioglio 2010; Wagner 1997)

Morphologically cyanobacteria range from simple unicellular species to colonial ones, which form complex filamentous forms that posses several highly differentiated cell types, i.e. heterocysts & akinetes (Rippka 1988). However, they do not attain any higher level of complexity. Sexual reproduction is entirely unknown among these organisms. The principal photosynthetic pigment is chlorophyll-*α* (chl-*a*). The accessory pigments, i.e. involved in light harvesting, are biliproteins, and they are usually organised into large, membrane peripheral antenna complexes known as phycobilisomes.

Cyanobacteria are remarkably tough and resilient organisms. They are found in almost every conceivable habitat where light is available, ranging from freshwater, marine and terrestrial environments to extreme hot springs and even on the surfaces of rocks in Antarctica and hot deserts.

Cyanobacteria are capable of adopting several different modes of metabolism with respect to their carbon and energy sources. As yet, all identified cyanobacterial species are able to grow in the light with CO_2 as the principal, if not the sole, carbon source. However, many species when provided with organic compounds in addition to carbon dioxide, may utilise the organic carbon in cellular processes, as for instance *Synechocystis* sp. PCC 6803 (Anderson & McIntocsh 1991). The extent to which the

organic substances are used in cell's metabolism varies widely from negligible amounts to the situation where all of the carbon molecules in a newly synthesised cell have derived from the organic substrate. Remarkably, some cyanobacterial species, as *Anabaena variabilis* ATCC 29413, have been reported to be capable of growth in the dark, in media containing organic substrates (Mannan & Pakrasi 1993). Presumably, under these particular conditions, the organic compounds are utilised as both, source of energy as well as source of carbon.

The variety of metabolical lifestyles, coupled with the relative ease of genetic manipulation and other advantages described below, has led to the adoption of cyanobacteria as model organisms in studies of important biological processes, including nitrogen and carbon fixation, light regulated gene expression, chromatic adaptation and cellular differentiation etc. Yet, their ability to perform oxygenic photosynthesis in a similar manner to higher plants in combination with a number of advantages as model systems resulted in wide use of cyanobacteria from studying the structure and function of photosynthetic apparatus and the thylakoid membranes.

Cyanobacteria as model organisms exhibit a series of advantages: (i) complete genome sequence is now available for numerous cyanobacteria, e.g. *Synechocystis sp* PCC6803 (Kaneko *et al.,* 1995) hereafter as *Synechocystis* 6803; *Thermocynechococcus elongatus* BP-1 (Nakamura *et al.,* 2002) (http://www.kazusa.or.jp/cyano/), *Synechococcus elongatus* PCC7942 (http://genome.jgi-psf.org/finished_microbes/synel/synel.home.html), henceforth *Synechococcus* 7942, and many others. The small genome sizes and the availability of their sequences, the relatively short generation time, all these, in combination with the ability for natural uptake of exogenous DNA (Shestakov & Khyen, 1970; Vermaas 1996) in certain species, have transformed the laborious techniques of cloning and gene modification into routine procedures. Nevertheless, cyanobacteria are polyploid organisms, and under laboratory growth conditions seem to contain up to ten genome copies per cell (Golden, *et al.,* 1986; Labarre *et al.,* 1989). This may represent a problem when isolating a recessive mutant, inasmuch as many rounds of restreaking are required to segregate a mutant, and full segregation is not always achievable. (ii) Certain cyanobacteria such as *Thermosynechococcus elongatus* BP1, are very thermophilic organisms; a feature that makes them an excellent model for crystallographic studies (Jordan *et al.*, 2001; Zouni *et al.,* 2001; Guskov *et al.,* 2009).

Nevertheless, molecular genetic studies on this organism have been hampered due to lack of standardised gene manipulation techniques, such as a means of transformation. (iii) In contrast to chloroplast where thylakoid membranes are organised into grana and stroma lamellae, cyanobacteria have much less convoluted conformation of their thylakoid membranes (TM). Furthermore in some species such as *Synechococcus* 7942, *Thermosynechococcus elongatus* BP1 or *Dactylococopsis salina,* the thylakoid membranes are aligned in concentric cylinders extending the full length of the elongated cell. Such species with elongated cells and even distribution of TM, are perfect model organisms to investigate the dynamics of the thylakoid membranes (Mullineaux *et al.,* 1997). Use of Fluorescence Recovery After Photobleaching (FRAP), allows the detection of TM components diffusion as well as the measurement of the coefficient rate of diffusion.

4. Basic principles of energy storage

The essence of all electron transfer reactions, relies on the ability of chlorophyll-like pigments to absorb light, leading the molecule to a higher energy level known as the excited state. At this point, the energy of an excited chlorophyll molecule can either be transferred to a nearby pigment by resonance energy transfer or decayed by emitting light or heat, a process known as non-photochemical quenching (NPQ). Migrations of electronic excited states, from one molecule to another, consist the basic principle of antenna functioning. However, the absorption of light by a pigment also changes its chemical properties. In particular, light absorption significantly lowers its redox potential (E_0), creating thus an extremely strong reducing agent that readily gives up an electron to a nearby acceptor. This electron transfer takes place only in reaction centres and consists the primary step of energy conversion from pure energy of excited states to chemical changes in molecules. The initial electron transfer event is followed by a series of rapid secondary, stabilising reactions that lead to separation of negative and positive charges across the membrane. This basic mechanism applies to all chlorophyll-based types of photosynthesis.

However, if the oxidised donor and the reduced acceptor are not regenerated, the electron transfer cannot take place a second time and the process comes to a halt. Therefore, to restore the system to the stage prior to the excited state, electrons must be donated to the primary donor and extracted from the final acceptor. To accomplish this

task, photosynthetic organisms have developed two conceptually different ways, namely cyclic and linear (non-cyclic) electron transfer pathways. The main feature of the cyclic electron transfer is that there is no net oxidation or reduction of any substrate. The electron from the primary donor, a special chlorophyll molecule in the reaction centre, following downhill transfer reactions, returns to the oxidised chlorophyll restoring it to the initial ground state. This electron transport pathway also pumps protons (H^+) across the membrane, generating a concentration gradient required for ATP synthesis. For this reason, the cyclic electron flow is often called cyclic photophosphorylation. It produces neither oxygen (O_2) nor NADPH. The best example of organisms operating in this mode, are purple bacteria (reviewed in Blankenship 2002). Green non sulfur and heliobacteria also carry out cyclic electron transfer although heliobacteria differ significantly in this aspect by using only membrane bound cytochromes (Kramer *et al.,* 1997). In contrast, linear electron transfer pathway requires two substrates; an electron donor that feeds electrons to the system and an acceptor that ultimately becomes reduced. It also involves two types of photosystems, referred to as photosystem-I (PS-I) and photosystem-II (PS-II), which are always arranged in series. Although both types of reaction centres can individually operate in cyclic fashion under certain conditions, linear electron transfer consists the major pathway employed by all oxygenic photosynthetic organisms and ultimately leads to oxidation of water and reduction of $NADP^+$.

5. The Z-scheme

When the overall reaction of photosynthesis was established and expressed in form of a chemical equation the scientific attention was switched to elucidating the detailed picture of the photosynthetic mechanism. The first ideas upon this matter were very simplistic proposing that the final product of photosynthesis, the carbohydrate was actually a fairly straightforward process involving chlorophyll, water and carbon dioxide. Of course now, it is well known that those early mechanistic ideas are not valid and that the overall equation of photosynthesis is deceptively simple. In fact, photosynthesis consists a very complex set of well-coordinated and regulated series of physical and chemical reactions that could be separated into two major group. The first set of reactions is the light driven electron transport through thylakoid membranes coupled with ATP production. The other half of reactions that takes place in stroma is

largely enzymatic and it is leading to reduction of NADP and assimilation of carbon dioxide. These two groups of reactions are traditionally called Light and Dark reactions respectively. However, this nomenclature, based on seminal experiments of R. Emerson and W. Arnold back in 1932, is somewhat misleading, in that all the reactions are ultimately driven by light. Yet, strictly speaking the only light induced step is that of photon absorption. Moreover, the dark reactions that in fact never occur at night, involve enzymes that are regulated by compounds produced by light-driven processes.

The light-induced non cyclic electron transfer pathway in oxygenic photosynthetic organisms is a multistep process involving two types of photosystems working in series, a cytochrome complex and a number of intermediates. Although not intrinsic, the molecules of H_2O and $NADP^+$ are essential elements of this pathway. The water is the reducing agent that provides both electrons to restore the oxidised primary electron donor, and protons (H^+) to create electrochemical potential across the membrane required for ATP synthesis. $NADP^+$ is the final electron acceptor and when reduced to NADPH, consists the major reducing agent in carbon assimilation processes. The ultimate products of the light-driven electronic reactions in oxygen evolving photosynthetic organisms are ATP (from photophosphorylation of ADP) and NADPH (from photoreduction of $NADP^+$)

The energetic picture of this process, with all the compounds of the electron transfer chain arranged according to their redox potential, is called Z-scheme. Although seemingly self-evident today, the idea of two reaction centres working concurrently but in series emerged very slowly.

Proposed by Hill and Bendall in 1960, the Z-scheme outlines the concept of two photosystems arranged not in parallel but in series, so the product of one reaction centre becomes the substrate for the other. Thus, if the absorption spectra of the two photosystems are not the same, the Z-scheme easily explains the puzzling results of the red drop and enhancement effect. The slightly latest experiments of antagonistic effect of light wavelength on cytochrome oxidation (Duysens *et al.,* 1961) completed the basic framework of light induced electron flow in oxygen evolving organisms and turning the Z-scheme into fundamental feature of modern understanding of photosynthesis. PS-II reaction centre that preferentially absorb at shorter wavelengths, oxidises water and

reduces cytochrome, while photosystem-I absorbing at longer wavelengths oxidises cytochrome and reduces $NADP^+$ (Duysens *et al.,* 1961; Duysens, 1989)

Almost inevitably, with the discovery of new components, the overall picture of Z-scheme has grown in details. The 3D resolution of its major components added even more to the complexity, such as binding sites of its cofactors and even allocation of roles to precise amino acids.

The modern understanding of the Z-scheme (reviewed in Blankenship 2002) suggests that the primary and secondary / stabilising charge separation reactions occur in the reactions centres embedded into the TM. The moment of initial conversion of solar power into chemical energy is, when the special pair of chlorophylls known as P_{680} in PS-II and P_{700} in PS-I transformed into strong reducing agents upon excitation, readily gives up an electron to a nearby electron acceptor. Because the generated ion-pair (oxidised P_{680}^+/P_{700}^+ and reduced acceptor P^-) is in a very vulnerable state, a series of extremely rapid secondary reactions takes place leading to stabilisation of the system by spatial separation of negative and positive charges. In case of P_{680}, a tyrosine residue of the PS-II "D1" subunit, acting as a bridge between the water oxidising manganese cluster, re-reduces the oxidised P_{680}^+. Electrons removed from water by PS-II are destined, through a series of intermediates, to re-reduce the oxidised P_{700}^+ of PS-I that eventually after a second light driven electron transfer step reduces NADP to NADPH.

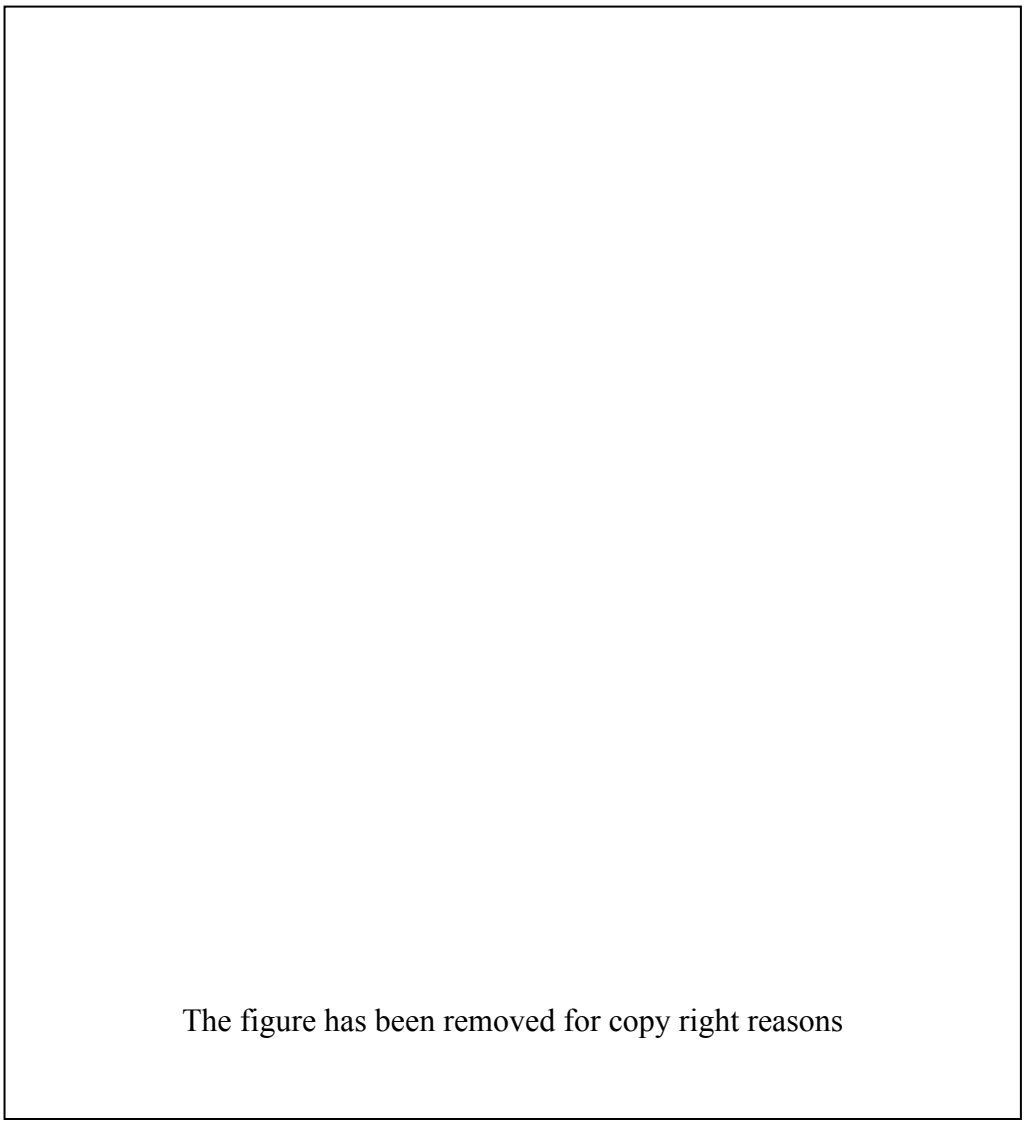


Figure I.2. The Z-scheme & electron transport pathway from water to NADP$^+$. (A) The 'Z' scheme of linear photosynthetic electron transport and redox potential diagram for the photosystems and components of the electron transport chain. Reproduced from: (http://www.answersingenesis.org/tj/images/v17/i3/photosynthesis_fig6.jpg). (B) Electron transport pathway from water to NADP$^+$ (Nicotinamide Adenine Dinucleotide Phosphate, oxidized form) and photophosphorylation of ADP. Reproduced from: (http://photoscience.la.asu.edu/photosyn/photoweb/electron-transfer.jpg)

6. Photosynthetic apparatus

The conversion of light energy into physiologically available chemical energy is accomplished by a group of membrane embedded protein-cofactor structures known as reaction centres or photosynthetic systems (simply photosystems – PS). Each of them contains a central catalytic domain, henceforth referred to as Reaction Centre (RC) and Light-Harvesting Complex (LHC).

All types of reaction centres identified to date share two characteristic features: they are all embedded within lipid bilayers in vectorial fashion and carry out light driven electron transport leading to charge separation across the membranes. Thus, the early stages in all kind of tetrapyrrole-based photosynthesis occur in reaction centres and are membrane-based processes. In contrast, the later stages of photosynthesis occur in the aqueous phase and are mediated by freely diffusible enzymes.

The thylakoid membrane in all oxygen evolving photosynthetic organisms is vesicular and defines a closed space (thylakoid) where the outer and inner spaces are known as stroma and lumen respectively. It is not known why the photosynthetic membranes form such convoluted structures.

An essential feature of all TM integrated complexes is that they are always oriented the same way with respect to the stromal and the lumenal sides of the membrane. Thus in oxygenic photosynthesis the acceptor side of the reaction centres is always facing the stroma while the donor side is oriented towards the lumen.

In eukaryotic organisms photosynthesis takes place in subcellular semi-autonomous organelles known as chloroplasts. In fact, chloroplasts have derived from photosynthetic cyanobacteria-like cells that were integrated long time ago into the host eukaryotic cell and eventually lost their independence through the process of endosymbiosis (Margulis 1996) Even today, chloroplasts retain part of their bacterial character, including their own DNA. Chloroplasts have an extensive and complicated system of photosynthetic membranes that contain most of the pigments, proteins and cofactors that are involved in the early, light-induced electron transfer reactions. Fixation and reduction of CO_2 are enzymatic reactions, and occur outside the membranes in the surrounding aqueous space of stroma.

6.1. Pigments - Antenna complexes

Pigments are molecules that selectively absorb and reflect light and as result of that appear colourful. Although there are numerous pigments found in different photosynthetic organisms and they play various roles, all pigments share two common structural characteristics; at least one ring structure and alternating single and double bonds (conjugated double bonds) between carbon atoms.

Pigments are vital compounds of all photosynthetic organisms but not every pigment carries out photochemistry. An early discovery from Emerson and Arnold back in 1932 that startle the scientific world of the time. In their seminal experiments, driven by the urge to understand the quantum requirement for the production of one molecule of O_2, i.e., how many quanta of light and the number of chlorophylls required to produce a single oxygen molecule, they calculated that nearly 2400 chlorophyll molecules were needed to capture 8 photons and generate one molecule of oxygen. To interpret the data, they introduced the concept of 'photosynthetic unit' where the vast majority of pigments have light harvesting functions, and as it has been established later, these molecules collectively absorb and transfer the energy to a special pair of chlorophylls in the reaction centres where the actual photochemistry takes place.

In order to increase the range of absorption spectra and maximise the efficiency of light harvesting, photosynthetic organisms use a wide range of pigments. In terrestrial plants, the major pigments carrying out light harvesting, are chlorophylls *a* and *b*. The polypeptide constituents of these light harvesting complexes are a group of related proteins, often called chlorophyll *a/b* binding proteins (Cab – Chlorophyll *a* and *b*) [Chitnis *&* Thornber 1988; Green *et al.,* 1991; Murphy 1986]. Genes encoding Cab polypeptides constitute a nuclear, multigene family [Chitnis *et al.,* 1988; Green *et al.*, 1991] that is regulated by light [Thompson *&* White. 1991; Tobin *&* Silverthorne*,* 1985].

In marine algae such as diatoms, dinoflagellates, brown algae and chrysophytes the major antenna complex contains xanthophylls such as fucoxanthin or peridinin and chlorophyll *a* and *c* [Fawley & Grossman 1986, Friedman & Alberte 1986, Larkum & Barrett 1983]. Constituent polypeptides of these complexes exhibit some homology to the Cab proteins [Grossman *et al.,* 1990], which may eventually be related to the chlorophyll binding functions of both protein families.

In cyanobacteria and eukaryotic red algae, the large peripheral antenna complexes known as phycobilisomes (discussed in details below) utilize the water-soluble pigments bilins. The constituent polypeptides of these antennas, called (phyco)biliproteins, are structurally very different from the polypeptides of other antenna complexes.

6.1.1. Antennas

Conceptually, antenna complexes resemble satellite dishes in that they collect electromagnetic waves (light), and then concentrate them into a receiver (Reaction Centre), where the "signal" (energy of excited electrons) is converted into a different form of energy (photochemical). The energy transfer that takes place in antennas is purely a physical process and involves migration of electronic excited states from one pigment to another. Structural main feature of antenna complexes is that they are pigment-protein interactions in which chlorophylls or other pigments are specifically associated with proteins. The only known exception is the case of chlorosomes [Blankenship *et al.,* 1995, Olson *et al.,* 1998] that are formed by direct pigment-pigment interactions and may contain up to 10,000 molecules of bacteriochlorophyll *c,d* or *e*. They are found in green photosynthetic bacteria that inhabit environments of extremely low light level.

Antennas are ubiquitous among photosynthetic organisms suggesting the importance of light harvesting systems to these organisms (Green 2003). In contrast to reaction centres (only two types), antennas are incredibly diverse. This structural diversity of antenna apparatuses suggests that they have derived not from one but from several independent evolutionary origins.

Despite their diversity, antennas can be broadly divided into integral membrane antenna complexes and peripheral antenna complexes. Proteins of the integral antenna complexes are embedded within the thylakoid membranes. This group, is itself quite diverse in terms of structure, pigment composition, and relative position in the energy transfer sequence. Thus, some of them such as CP47 and CP43 (subunits of Photosystem-II) for instance, are associated with the reaction centre core subunits and form an antenna within the core complex. The significance of some core antennas complexes is extended beyond the light harvesting functioning as is the case of CP47 which is the prerequisite for the assembly of Photosystem-II and photoautotrophic growth (Vermaas *et al.,* 1986, 1988). Accessory antennas as LHC-I /II (Light Harvesting Complex-I/II) of higher plants and many algae, are also integral membrane antenna complexes, but in contrast to core antennas, they are present in variable amounts, depending on the growth conditions. Yet, their physical arrangement with other antennas and reaction centres is not fixed in either time or space. They play an

important role in antenna regulatory processes as state transitions [Allen *et al.,* 1992, Allen & Nilson 1997, Allen & Forsberg, 2001].

Peripheral antenna complexes are associated with other membrane embedded components but do not themselves span the membrane. Into this group fall phycobilisomes (examined in details below) of cyanobacteria & red algae, and chlorosomes & FMO proteins (named after Mathews and Fenna who determined their structure, and Oslon who discovered the protein) of green photosynthetic bacteria [Blankenship *et al.,* 1995, Olson 1998, Matthews & Fenna, 1980]

Phycobilisomes

Phycobilisomes (PBS) are large, peripheral membrane antenna complexes found in cyanobacteria and the eukaryotic red algae [Gantt & Conti 1969; Gantt 1975, 1981; Edwards & Gantt 1971; MacColl 1998, Grossman *et al.,* 1995]. PBS are located on the stromal side of the TM and preferentially deliver the absorbed energy to PS-II. Under certain conditions nevertheless, they can transfer the excitation energy directly to PS-I [Mullineaux 1992, 1994; vanThor *et al.,* 1998]. The coupling of PBS to reaction centres in cyanobacteria appears to be regulated by DNA-binding proteins [Ashby & Mullineaux 1999].

They have a wide absorbance range (500-650 nm), which is very advantageous in changing environments, especially under light limited conditions.

Phycobilisomes are composed of biliproteins (pigment-proteins complexes), and linker polypeptides. Biliproteins are water soluble, structurally very different from the polypeptides found in other antenna complexes and absorb radiation of the visible spectra where chlorophyll-*a* has low absorbance. Produced in massive scale, they may consist up to 50% of the soluble proteins of the cell. All biliproteins are composed of two different subunits termed as α and β, and contain bilin chromophores (linear, open chain tetrapyrrole pigments), covalently bound to protein's cystein residues via thioether bonds. No metal ions are associated with bilins, although they can be attached to biliproteins [Cheng *et al.,* 1990, MacColl *et al.,* 1994] There are several types of bililiproteins: phycoerythrin (PE), phycoerythrocyanin (PEC), phycocyanin (PC) and allophycocyanins (AP), which differ in protein identity, number and type of associated bilins and in absorption spectra – an attribute that dictates to certain extent, the relative

position of the biliprotein in the PBS complex. The number and type of bilins pigments (phycocyanobilin, phycoerythrobilin, phycourobilin, phycoviolobilin) associated with a particular biliprotein is usually, but not always, invariant. Cyanobacterial and red algal biliproteins contain up to two different billions, although biliproteins with three different bilins for light harvesting have also been discovered in cryptomonads [Ducret *et al.,* 1994; Samsonoff & MacColl 2001]. Unlike their counterparts of cyanobacteria and red algae, biliproteins of cryptomonads are not organised into phycobilisomes and are located in the intrathylakoid space [Gantt *et al.,* 1971].

Linker polypeptides consist an essential part of PBS complexes [Tandeau de Marsac & Cohen-Bazire 1977]. Identified in cyanobacterial and red algal PBS, they may consist up to 15% of the total mass of the complex [Tandeau de Marsac and Cohen-Bazire 1977, Yamanaka *et al.,* 1978]. Most of them are colourless while some of them contain attached chromophores like biliproteins. Linker polypeptides govern the attachment of PBS to the thylakoid membranes and the assembly of the biliproteins [Tandeau de Marsac & Cohen-Bazire 1977, Lundell *et al.,* 1981]. Although linkers are not involved in energy absorption, in certain cases they modulate the absorption characteristics of some biliproteins [Glazer, 1985; Glazer *et al.,* 1985 a, b]. The linker-free and the linker-biliprotein complexes have different optical spectra. The modification of biliprotein absorbance (caused by interactions with linkers) in turn, improves the efficiency of energy transfer, both within the PBS and from it to the chlorophyll in TM [Yu *et al.,* 1981; Wendler *et al.,* 1986].

Despite the accumulated knowledge on the role of linker polypeptides in the assembly of PBS complexes, many aspects of the process remain still unclear. How do linkers accomplish their extraordinary specificity of disk to rod association, given that the process is highly influenced by the environment? What is the molecular nature of linker-biliprotein interaction? The biogenesis of other macromolecular complexes always requires the assistance of enzymes or chaperons, that as a rule, and in contrast to linker polypeptides do not remain associated with the mature complex. Since linker polypeptides are insoluble unless bound to biliproteins, it is very plausible that some chaperones at first place suppress the precipitation of linker polypeptides and second, hold them in an appropriate conformational state that allows the association with the particular biliprotein.

PBS model

Several, structurally different types of phycobilisomes, e.g., hemiellipsoidal, bundle-shaped, block-shaped, hemiellidiscoidal, are found among cyanobacteria and red algae [Glazer 1985; Grossman 1993; MacColl 1998; Mimuro *et al.,* 1999]. Cyanobacterial species with their biliproteins not organised into phycobilisomes have also been reported [Reuter *et al.,* 1994].

The most widely studied phycobilisomes are the hemidiscoidal type, depicted in Figure I.3. It is extensively found among cyanobacteria and it is the type of PBS present also in our model organism, viz., *Synechococcus sp.* PCC 7942. The hemidiscoidal phycobilisomes are composed of two major domains: the core and the rods, which together in lateral view, have fan like appearance. The core domain is predominantly composed of allophycocyanin, and contains ring like structures, which in three dimensions resemble cylinders. The rods consist of stacks of disks, radiating from the core and each rod may contain up to 6 disks depending on the organism and the impact of the environment (light intensity & quality, nutrients availability, etc.). The disks of the rods are either, exclusively PC or both PC and PE, but the disk adjacent to the core is invariably phycocyanin.

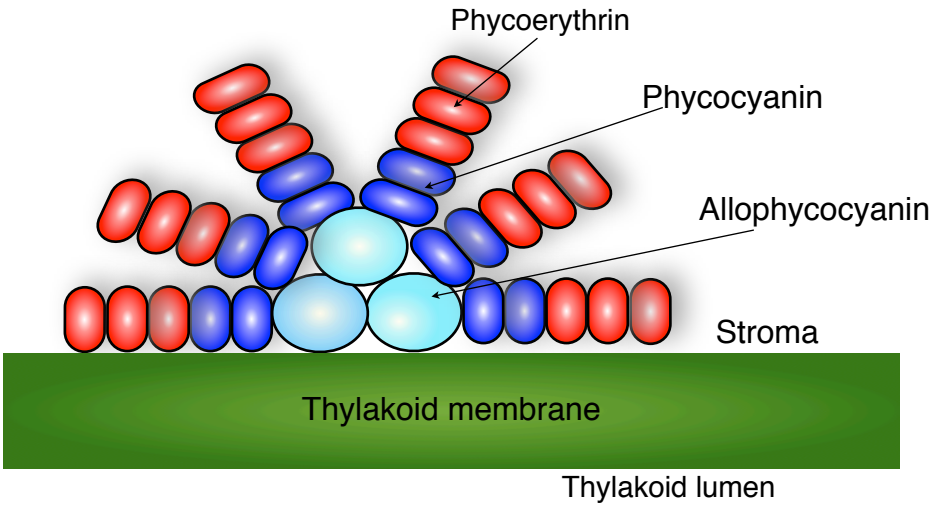


***Figure I.3*. Structural model of a typical hemidiscoidal phycobilisome**. The three cylinders-like at the core of the antenna represent allophycocyanin, whereas the six rods composed from phycocyanin and phycoerythrin give the phycobilisomes its fan-like appearance.

Arrangement of phycobiliproteins and energy transfer

Phycobilisomes are dynamic systems. Despite their bulky appearance they are capable of moving very fast (diffusion coefficient $3x10^{-10}$ cm^2 s^{-1} at 30 ^{0}C) [Sarcina *et al.,* 2001] on the surface of thylakoid membranes. They can also alter their composition according to the environmental changes ensuring maximum absorption over a wide range of visible spectra and maximum efficiency of energy transfer from the point of absorption towards the reaction centre. The deposition of the biliproteins within the PBS and the organisation of its many bilins provides efficient energy flow from the highest energy PE (or APE) and through the intermediate energy PC to the lowest energy AP from which the energy is ultimately delivered to chlorophyll-α in the thylakoid membrane.

6.1.2. Chromatic adaptation

One of the distinguishing characteristics of cyanobacteria is their plasticity; an extraordinary ability of adaptation to constantly changing environment. Low light intensities for instance result in increase of phycobilisome amounts and the length of the rods. Such result can also be stimulated when cyanobacteria predominantly receive photosystem-I light that is believed to help balance the electron flow between the two photosystems [Mandori & Melis 1985, 1986]. The ratio of photosystem-I to photosystem-II depends upon the intensity of incident light and changes from nearly a unit in saturating light to several-fold increase in favour to photosystem-I in dim light environment.

During nitrogen or sulfur starvation, phycobilisome degradation is an ordered process as it allows recycling of amino acids into other proteins, essential for cell homeostasis.

The abundance as well as the composition of PBS depends greatly on environmental conditions. The ability of certain cyanobacteria to alter the composition of their PBS in response to light quality is termed chromatic adaptation and was first reported by Engelman & Gaidukov over a century ago. During the phenomenon, the pigment which absorbs the incident wavelengths of light more strongly becomes predominant. The chromatically induced change in the colour of cells is largely if not entirely attributable to changes in the ratio of PE to PC. Phycocyanin predominates after growth in red light (RL), while phycoerethrin in Green light (GL). Although synthesis of PE for chromatic adaptation is regarded as necessary, it is not a sufficient cause since several PE-producing cyanobacteria do not adapt chromatically. The phenomenon is not known to

occur in red algae. Based on chromatically induced changes in pigmentation, cyanobacteria can be classified into three main groups: species with no changes in PBS composition; those that modify the PC content only; and cyanobacteria that change the ratio of PC to PE. The last case, where the levels of PC and PE are adjusted in opposite direction is known as complementary chromatic adaptation.

6.2. Reaction centers

Reaction centres are integral membrane energy-transducing devices that capture and transform the pure energy of sunlight into chemical changes in molecules. All reaction centres found to date carry out light driven electron transport; yet the RC of purple bacteria are capable of coupling the electronic reactions with proton (H^+) translocation. Structurally, a RC consists a multisubunit protein-pigment-cofactor complex, which threads the lipid bilayer in vectorial fashion, ensuring that the complex is always oriented the same way with respect to the stromal and lumenal side of the TM. In addition to the protein subunits, reaction centres also contain a number of non-covalently associated cofactors, such as quinones, pheophytins (metal free tetrapyroles), metal ion (most commonly Fe^{2+}), and iron sulfur clusters (Fe-S).

An essential prerequisite for rapid and efficient transfer of energy and electrons, is the strict positioning of subunits within the complex, and the precise attachment of all electron transport intermediates to the proteins. From that point of view, some of the subunits in the RC function as a sophisticated scaffold ensuring optimum distance and orientation of these elements, and this in turn is what creates the network of excitonically linked pigments and other cofactors.

Unlike the diverse antennas, all reaction centres belong to two distinct groups, conveniently classified by the respective terminal electron acceptors. Type-I RC utilise iron-sulfur (Fe_4S_4) clusters as their terminal electron acceptors. This group, also known as iron-sulfur type, includes the photosystems of anoxygenic heliobacteria (PS-H) and the green sulfur bacteria (PS-C), as well as the PS-I of oxygen evolving photosynthetic organisms (higher plants, green algae and cyanobacteria) (Buttner *et al.,* 1992, Liebl *et al.,* 1993; Blankenship 1995). The abbreviations H and C (in PS-H and PS-C) derive from the genera Heliobacillus and Chlorobium respectively. The second group of RC complexes, referred to as type-II, uses quinones as their terminal electron acceptors and

hence the alternative name quinone-type RC. Into this group fit the RC of green filamentous bacteria (GbRC), purple bacteria (PbRC) and photosystem-II (PS-II) of all oxygen evolving photosynthetic organisms (Barber 1992; Ort & Yokum 1996). Whereas all anoxygenic photosynthetic bacteria have only a single RC, either type I or type II, oxygen evolving organisms possess both types reaction centres I & II, that are working in series and ultimately oxidise water and reduce $NADP^+$ at the two extremes of the electron transport system.

	Type I RC				**Type II RC**	
	Anoxygenic		*Oxygenic*		*Anoxygenic*	
	Photosystems					
Organism	Heliobacteria	Green sulfur bacteria	Cyanobacteria, prochlorophytes, green algae, higher plants		Purple bacteris	Green filamentous bacteria
Abbreviation	HB	GSB			PB	GB
RC-complex	PS-H	PS-C	PS I	PS II	PbRC	GbRC
Core proteins	$(PshA)_2$	$(PscA)_2$	PsaA/PsaB	D1/D2	L/M	L/M
Antenna proteins	$(PshA)_2$	$(PscA)_2$	PsaA/PsaB	CP43/CP47	none	none

Table I.1. **Reaction Centres found in chlorophyll-based photosynthetic organisms.** All oxygen evolving photosynthetic organisms contain two reaction centres, Photosystem-I and photosystem-II, arranged in tandem, so the product of one becomes substrate for the other. In contrast, all anoxygenic photosynthetic organisms possess only one reaction centre either, type-I or type-II. Core protein subunits in type-I RC contain a RC and an antenna domain. In contrast, the PS-II core antenna proteins are distinct from the core D1 and D2 subunits. Apart from the D1, D2, CP43, and CP47, none of the PS-II subunits have been shown to bind Chl-*a* of the core antennas or electron transfer cofactors.

Intensive studies using various biochemical, biophysical, computational and molecular biology techniques have been carried out over the past few decades in order to elucidate the structures and functioning of photosynthetic systems. Although the RC found in all photosynthetic organisms vary greatly in composition and complexity, they exhibit important similarities in both electron transport system and structure of the core protein subunits. In all cases, the electron transfer chain utilises similar set of cofactors yet exhibiting strong analogy in their arrangement. In particular, the set of cofactors present

in all RC includes: a pair of (bacterio)chlorophyll molecules constituting the primary donor (limitations concerning P_{680} are discussed below); a second pair of (bacterio)chlorophylls known as accessory chlorophylls; a pair of tetrapyrroles (pheophytins or chlorophylls) as at least one of them is redox active; two quinones that either one or both of them are involved in electron transfer reactions; and finally an iron species (in type-I RC as a non heme Fe^{2+}, or sometimes Mg^{2+} Arnoux *et al.,* 1995) and in type-II RC as Fe_4S_4 clusters). Also, the constituent core protein subunits of all RC form a (pseudo)symmetric scaffold of two sets of five transmembrane α-helices. In turn, this hetero(homo) dimer binds and arranges the electron transfer cofactors into two membrane-spanning branches around a central axis perpendicular to the plane of the membrane.

The structural similarities between the RC-cores of both types (I and II), and the striking analogy in the electron transport system, simply confirm the long-standing hypothesis of a common evolutionary origin for all RC-complexes (Blankenship, 1992; Barber, 1992).

6.2.1. Photosystem-II (PS-II).

Introduction

Photosystem-II is a membrane embedded pigment-protein complex found in oxygen evolving photosynthetic organisms (higher plants, green algae and cyanobacteria) which utilising the solar power, splits the water into O_2, protons (H^+) and electrons (Barber, *et al.,* 1997; Hankamer, *et al.,* 1997). The ability of this complex photosynthetic enzyme to drive one of the most (if not the most) thermodynamically demanding reactions known to occur in nature, namely the oxidation of water, makes it unique among any other protein complexes involved in bioenergetic reactions of energy converting systems found in plants, animals and bacteria. Yet this reaction (water oxidation) takes place on an enormous scale, and gives rise to molecular oxygen in the atmosphere, vital for the sustenance of a vast number of living organisms on this planet. Furthermore, another characteristic feature of PS-II adds even more to its uniqueness and complexity – unlike any other reaction centre this photosynthetic enzyme has an unusually high rate of turnover. Light, the source of energy that drives photosynthesis, when in excess, causes irreversible damage to PS-II structural sub-units, rendering the complex nonoperational (for details see below: photoinhibition).

Electron transport.

PS-II utilising the solar energy carries out the reactions of water oxidation and reduction of plastoquinone (PQ), summarised in equation 1. This is why this reaction centre is often referred to as water-plastoquinone oxyreductase.

$$2H_2O + 2PQ \rightarrow O_2 + 2PQH_2$$

***Equation 1.1*:** The overall reaction catalysed by PS-II. PQ refers to the oxidized plastoquinone, and PQH_2 is the fully reduced plastoquinone (known as plastoquinole) that dissociates from PS-II D1 subunit and migrating through the thylakoid membrane, finally reduces the cytochrome cyt-b_6f complex. There are 6-8 quinone molecules per PS-II, cumulatively called Plastoquinole Pool which serves two important functions: first it feeds electrons to PS-I via cyt-b_6f complex to reduce the $NADP^+$ and second it carries protons H^+ from the stromal to the lumenal side of the thylkoids generating thus electrochemical potential required for the ATP production.

The reactions occurring in PS-II RC of eukaryotes (higher plants and green algae) and cyanobacteria are very similar (Ort & Yocum 1996).

The energy of light, trapped by either outer antenna systems (LHC-II in higher plants and green algae or PBS in cyanobacteria) or core antennas CP43 and CP47 (present in all oxygen evolving photosynthetic organisms), is ultimately passed to P_{680}, which is thought to be a pair of chlorophyll-*a* molecules, and consists the primary electron donor. These pigments are chemically identical or nearly so to the other chlorophylls found in antennas, but their environment gives them unique properties and together they form the photoactive pigment called P_{680}, after the wavelength maximum of its absorbance band (limitations about P_{680} are discussed below). The excited state of P_{680} designated as P_{680}*, an extremely strong reducing agent, donates within a few picoseconds a single high-energy unpaired electron to a nearby molecule of pheophytin (Pheo), thereby forming the radical pair, P_{680}^+ $Pheo^-$. Because the system of two physically adjacent ions such as P_{680}^+ and $Pheo^-$ is very susceptible to recombination, and concomitant loss of energy, rapid stabilisation reactions take place leading to spatial separation of negative and positive charges. Indeed, within a few hundreds picoseconds, the electron from $Pheo^-$, following downhill reactions is passed to electron stabilising acceptor quinone Q_A, reducing it to semiquinone form Q_A^-. On milliseconds timescale Q_A^- passes the electron to the nearby secondary quinone Q_B. The rate of electron transfer from Q_A^- to Q_B depends on the redox state of Q_B and is aided by a non-heme iron. The removal of this metal ion does not prevent the electron flow between the two quinone carriers (Q_A

& Q_B) but just slows down the process. Although, these two quinone molecules are chemically identical, they exhibit distinct properties because of differences in their protein environment. The Q_A molecule, which remains tightly associated with the PS-II D2 core subunit, is able to accept and transfer only one electron at a time. Unlike its counterpart, the secondary quinone Q_B is loosely bound to the D1 core subunit, and accepts sequentially, one at a time, two electrons in total. When doubly reduced, Q_B^{2-} is protonated by two hydrogen atoms (H^+) from the stroma and at this stage, the fully reduced protonated quinone Q_BH_2 (called plastoquinone - PQH_2), dissociates from PS-II and by diffusing through the lipid matrix ultimately reduces the cytochrome cyt-b_6f complex. Because Q_A functions as one electron gate, oxidation of the semiquinone Q_A^- to initial Q_A form makes it capable of going through the reaction cycle again. The Q_B^- state is relatively stable, lasting until the next photon excites P_{680}. To form the fully reduced plastoquinone, PS-II must turn over twice, since the photochemistry of the RC is one electron at a time. The passage of PQH_2 towards cyt-b_6f complex followed by its oxidation from Fe-S Rieske protein of the cyt-b_6f complex consists the slowest step of the entire sequence of electron transfer reactions (timescale in milliseconds).

After the formation of semiquinone Q_A^-(nanoseconds), the oxidised primary donor P_{680}^+ is re-reduced to P_{680} by an electron from WOC (water oxidising complex) via a redox active D1-subunit tyrosine residue (D1-Tyr161) called Y_Z or Z. It is generally believed that Y_Z is deprotonated, forming a neutral radical, designated as $Y\blacksquare_Z$. The data (Schilstra, *et al.,* 1998) suggests that to complete the reduction of P_{680}^+, proton migration is essential. The numbering of amino acids (D1-Tyr161) corresponds to protein sequence of the cyanobacterium *Thermosynechococcus elongatus*. In similar position on D2 core subunit there is another tyrosine residue known as Y_D (or D), which is symmetrically positioned to Y_Z of D1. Although this particular amino acid Y_D is usually found in the oxidised form, which is relatively stable, it appears not to be involved in the mainstream of electron flow. It is oxidised by P_{680}^+ in the first photochemical turnover after long dark period.

Concurrent with the oxidation of the semiquinone (Q_A^-), the neutral tyrosine radical $Y\blacksquare_Z$ extracts an electron and most likely a proton from the Water Oxidising Complex (WOC) that binds the two substrate molecules of water. Unhindered electron flow from P_{680} to PQ, combined with energy transfer from antennas to P_{680} or direct photon absorption by

the primary electron donor, resets the cycle $P_{680} \rightarrow P_{680}^* \rightarrow P_{680}^+$, which in turn enables sequential extraction of electrons from the WOC via Y_Z. Four such photochemical turnovers help the manganese cluster to form a total of four oxidising equivalents required to oxidise two water molecules into molecular oxygen.

P_{680} – the primary donor

Despite the analogy in electron transport systems and the structural architecture between PS-II and PbRC, there is a number of considerable differences concerning the redox chemistry on the donor side of the complex. The first is the identity of the donor species that re-reduces the oxidised primary donor. In purple bacteria, the photooxidised radical cation P_{870}^+ is re-reduced by a cytochrome, while P_{680}^+ in PS-II returns to its initial state P_{680} by electrons extracted from water and donated by the tyrosine residue Y_Z. The second major difference lies in the very core of the reaction centres, the primary donors themselves. Unlike any other primary donor, P_{680} is unique in that, it achieves unusual high redox potentials (Em~ 1.2V). How this extreme redox potential is achieved remains obscure.

Furthermore, the identity of P_{680} is still an issue under debate; does it consist a 'special pair' of chlorophylls or it must be regarded as two monomeric chlorophyll molecules? It has been mentioned earlier on, that until recently, P_{680} was considered, as a "special pair" alike the primary donors in the rest of the reaction centres (e.g. P_{700}, P_{870} in PS-I and PbRC respectively). Consistent with the view of recent years that P_{680} is not a dimer (Durrant *et al.,* 1995), the data from X-ray crystallography (Zouni *et al.,* 2001; Guskov *et al.,* 2009) show that the chlorophylls of P_{680}, henceforth referred to as P_{D1} and P_{D2} are not close enough (distance between Mg-Mg ~10Å) to form a special pair. The excitonic coupling is weak and P_{D1} & P_{D2} may be regarded as monomeric chlorophylls suggesting that the unpaired electron in P_{680}^+ is located on one of them. This characteristic feature of P_{D1} and P_{D2} chlorophylls (reduced coupled and increased monomeric) could be due to the requirement of P_{680} to generate high redox potential (Barber and Archer, 2001). Resent experimental evidence (Prokhorenko & Holzwarth, 2000) suggests that the initial charge separation could occur from the accessory chlorophyll Chl_{D1} and the generated radical cation Chl_{D1}^+ may then oxidase P_{D1} or P_{D2}. If this proposition is correct then all four chlorophylls: P_{D1}, P_{D2}, Chl_{D1} and Chl_{D2} are high potential species, and PS-II therefore is novel in terms of primary electron transfer.

Arrangement of cofactors

The cofactors of the light-induced electron transport system (ETS) in PS-II RC, form two branches, symmetrically organised along the pseudo-C2 axis. All of them are bound to either D1 or D2 subunits. Towards the lumenal side there are two chlorophyll molecules referred to as P_{D1} and P_{D2}, with their head groups arranged in parallel and perpendicular to the membrane plane (Zouni *et al., 2001;* Guskov *et al., 2009)*. They may represent P_{680}. Towards the stromal side, there are two additional chlorophyll-*α* molecules, known as accessory chlorophylls Chl_{D1} and Chl_{D2} and their planes are slanted against the membrane plane. Above the two chlorophylls, there are two pheophytin molecules $Pheo_{D1}$ and $Pheo_{D2}$ followed by the two quinones Q_A and Q_B. Unlike PbRC, photosystem-II possesses two extra chlorophyll-*α* molecules ($Chlz_{D1}$ and $Chlz_{D2}$) which are believed to play a role in energy, transfer from CP43 and CP47 towards P_{D1} and P_{D2}. Due to large separation of CP43 and CP47 from P_{680}, it is plausible that the route of excitation energy transfer may involve the combinations $Chlz_{D1}/Chl_{D1}$ and $Chlz_{D2}/Chl_{D2}$. The pseudo-C2 symmetry of the cofactors arrangement is broken by the cytochrome-b_{559}.

Side or cyclic electron transfer reactions

Unlike photosystem-I where cyclic electron transfer reactions are normal and have been documented for decades, photosystem-II cyclic electron flow until recently has been elusive.

The side reactions, involving several components all of which are part of PS-II (Hanley *et al.,* 1999), function primarily as a safety valve to prevent photodamage. Because they occur on the milliseconds timescale they do not compete with the mainstream electron flow. The cyclic electron transfer reactions in PS-II are only one of several levels of an overall protection mechanism operating not only to prevent but to repair any damage as well. Other such safety schemes of the overall protective mechanism include the xanthophyl cycle (Demmig-Adams & Adams 1992; Demmig-Adams & Adams 1996; Horton *et al.,* 1996;), photorespiration (Osmond 1981; Kozaki & Tacked 1996; Park et al., 1996; Badger *et al.,* 2000), water to water cycle (Park *et al.,* 1996; Asada 1999) and the D1 repair cycle. If or when normal electron transfer from WOC via Y_Z towards $P680^+$ is interrupted then in the absence of a safety mechanism the accumulation of long lived and highly oxidising radical cation $P680^+$ will lead to detrimental, non specific

oxidation reactions inactivating the system (Barber & DeLas Rivas 1993). The side reactions probably do not function to significant degree under normal conditions, but they may be vital under stress conditions or during the assembly of photosystem-II and / or reassembly during the Repair Cycle.

The set of elements involved in side reactions include the high potential cytochrome-b_{559}, Chl_Z, β-carotene and the secondary quinone Q_B. Various electron transfer pathways involving cyt-b_{559}, Chl_Z, carotenoid and P_{680} are currently under discussion (Faller *et al.,* 2001 a & b; Tracewell *et al.,* 2001; Hanley *et al.,* 1999).

The sequence of events in the cyclic flow is believed to be as follows: the oxidised primary donor $P680^+$ is reduced by the nearby β-carotene molecule. The oxidised carotene radical is re-reduced either by an accessory Chl_Z or by the high potential cytochrome cyt-b_{559} but the pathway is still not certain. The oxidised Chl_Z^+ is re-reduced by cyt-b_{559}, which in turn, is reduced by an electron from the secondary quinone Q_B.

A common feature of all photoprotective mechanisms in photosynthesis is the presence of carotenoids. The protective function of carotenoids correlates to their ability to quench excited chlorophyll triplet states (^{3}Chl) (Frank & Cogdell 1996; Codgell & Frank 1987) which in turn prevents the formation of very reactive and toxic singlet oxygen ($^1O_2$) that otherwise would occur. The triplet state from chlorophyll is rapidly transferred to carotenoid and then dissipated as heat, returning the carotenoid to its ground state. This characteristic feature of carotenoids is essential for cells *in vivo,* and it is in agreement with the fact that the cells lacking these pigments are light sensitive (Romer *et al.,* 1995)

Water splitting device

The most remarkable similarity in electron transfer reactions occurring in PS-II and PbRC is at the acceptor side of the electron transport system with clear evidence for the pheophytin acting as an early electron acceptor, and the two quinone molecules operating as two electron gates, analogous to the function of Q_A and Q_B. Nevertheless, despite any similarities between these RC, there is a fundamental difference in the oxidising side, which makes PS-II so unique. This is the Water Oxidising Complex (WOC), also known as Oxygen Evolving Complex (OEC) composed of a cluster of four

manganese atoms. Calcium (Ca_2^+) and chloride (Cl^-) ions have been shown to be essential for oxygen evolution, but despite that their precise role has not been determined as yet. The oxygen evolving complex is located off centre towards the D1 protein (Zouni *et al.,* 2001; Guskov *et al.,* 2009) and its catalytic site is buried within the large protein mass attached to the lumenal membrane surface. However, the OEC and the chemical mechanism of water oxidation remain one of the most active research areas in the entire field of photosynthesis, and the picture is still growing in details.

During the water oxidation process, the manganese $(Mn)_4$ cluster passes through a series of five oxidation states referred to as S-state cycle (S_0 – S_4) (Kok, *et al.,* 1970). Every single photochemical turnover of PS-II RC leads the $(Mn)_4$ cluster to the next S-state generating an oxidising equivalent. In contrast to previous belief, the new data suggest that the four oxidising equivalents required for the oxidation of water and release of dioxygen, do not accumulate but are utilised throughout the S-state cycle. Yet, the catalysis of water oxidation requires the ions of calcium (Ca_2^+) and chloride (Cl^-), which are believed to be in close proximity to the manganese cluster (Debus, 1992).

Due to high affinity of atomic oxygen for electrons (midpoint redox potential Em=0.82V) the molecule of water consists an extremely poor source of electrons. Therefore the water oxidation is thermodynamically very demanding reaction and requires an oxidising agent stronger than the elemental oxygen. One of the most powerful electron acceptors known to exist in nature is the oxidised primary electron donor P_{680}^+. It has very high midpoint redox potential (estimated to be Em=1,17 V by Klimov et al., 1979) and thus allows removal of electrons from H_2O via Y_Z. In recent years though, it has been proposed that Y_Z (Em ~ 1.0V) is involved in the water oxidation process itself, as a hydrogen atom abstractor or in promoting proton coupled electron transfer from the WOC (Nugent *et al,* 2001)

The Hydrogen atom abstractor mechanism proposed by Babcock and co-workers (Hoganson and Babcock, 1997; Tommos & Babcock, 2000) suggests a well-synchronised removal of a H^+ as well as an electron from the two molecules of water at each step of the S-state cycle (proton release pattern 1-1-1-1 for S_0-S_4). The release of protons into the lumenal side of thylakoids takes place within ten of microseconds (μs) after the excitation of P_{680}. This means that proton release precedes the actual S-state

transition, and it is coupled to reduction of P_{680}^{+} and oxidation of Y_Z rather than to reduction of Y_Z and oxidation of water itself. Yet, other data (Schilstra *et al.,* 1998) confirm that to complete the reduction of P_{680}^{+}, proton migration is essential. Thus, formation of deprotonated neutral $Y\cdot_Z$ is regarded as fundamental, since this facilitates its subsequent reduction by an electron and a H^+ at each step of the S-state cycle. Essential moment in the proposed mechanism is that the system remains electro-neutral because the radical states formed on the substrate water intermediates are stabilised by the increased level of oxidation of the $(Mn)_4$ cluster.

There is a number of alternative propositions attempting to explain the mechanism of water oxidation. The "high valency scheme" (Vretos *et al.,* 2001) suggests that the electro-neutrality proposed by Hydrogen atom abstractor theory does not happen, and the S_1-S_2 step evolves only electrons. Indeed, other experimental work (Hanmann & Judge, 1999) has established 1-0-1-2 proton release pattern, confirming that the S_1-S_2 step does not release protons. However, despite the differences between the two proposed mechanisms, both schemes accept that: first, the two substrate water molecules are bound to the system at S_0 state with one water molecule linked to Ca_2^{+} and second, the deprotonated neutral $Y\cdot_Z$ functions as a proton as well as an electron acceptor.

Whatever the precise mechanism of water oxidation, one thing is clear that it must involve light-induced highly oxidising cofactors generated in the reaction centre. The unusually high redox potential of P_{680}^{+}, makes it so special since no other oxidised primary donor in photosynthesis achieves this high potential (usually in the region 0.4-0.6 V). It is still an enigma how chlorophylls-*a* of P_{680} achieve this remarkable redox potential and most importantly how PS-II manages to minimises the possibility of the long-lived P_{680}^{+} to oxidise its surrounding environment. These issues add more to the uniqueness of PS-II and still remain a bit of mystery.

PS-II: Structure – Composition

Advances in crystallization techniques along with X-ray crystallography and electron microscopy (EM) have revealed significant structural details of PS-II reaction centre (Rhee *et al.,* 1998; Hankamer *et al.,* 1999; Nield *et al.,* 2000; Zouni *et al.,* 2000; 2001; Rhee *et al.,* 2001; Kamiya & Shen 2003; Guskov *et al.,* 2009). Although, these recent data are of relatively modest resolution, they provide great support to the long standing

proposal that PS-II and PbRC share a common evolutionary blueprint. Nevertheless, despite the series of structural similarities, photosystem-II differs significantly from the PbRc. One such difference worth mentioning, is the presence of a tightly bound bicarbonate ion in photosystem-II, which appears to be associated with the non heme iron (van Rensen *et al.,* 1999). The overall similarity between these two RC breaks down on the oxidising side, namely the WOC. Yet, photosystem-II is by far more complicated photosynthetic system than its counterpart of purple bacteria (Hankamer *et al.,* 1997). Up to day, more than 25 distinct genes (referred to as *psb* genes) have been identified amongst oxygenic photosynthetic organisms, encoding proteins for PS-II (Barber *et al.,* 1997). Yet, there are other genes encoding for the outer antennas: *cab* genes (chlorophyll *a* / *b* binding proteins) in higher plants and green algae and *apc* & *cpc* genes encoding allophycocyanin and phycocyanin respectively in cyanobacteria and red algae (Glazer, *et al.,* 1994). The polypeptide complement of cyanobacterial PS-II is very similar to that found in eukaryotic organisms. Single particle analysis of isolated PS-II core complexes from spinach and the cyanobacterium *Synechococcus elongatus* have revealed that the RC in both species had almost identical structures and similar composition [Boekema *et al.,* 1995]. The PS-II constituent protein subunits have been named after the genes encoding them (PsbA – PsbZ) and are listed in Table I.2.

Photosystem-II in vivo exists as a homodimer of two entire photosystems associated with each other and forming a single large structural unit (Hankamer *et al.,* 1997). The fundamental question concerning PS-II structure-based function, whether the dimeric structure is a prerequisite for optimal water-splitting activity, is still under dibate. Although the dimer of two photosystems appears to be the most stable structural form, there is still insufficient evidence that PS-II is functionally dimeric, so each of the two RC appear to function independently of the other. Monomerization of isolated dimmers does not inhibit significantly the water oxidation process (Hankamer *et al.,* 1997)

The cyanobacterial photosystem II is composed of at least 17 distinct subunits (Zouni *et al.,* 2001; Guskov *et al.,* 2009). Only three of them are membrane-extrinsic proteins, located at the lumenal side of the RC, namely: cytochrome-c_{550} (PsbV), PsbU and the manganese-stabilizing protein PsbO present in all oxygen evolving organisms. The membrane associated constituent polypeptides of photosystem-II are: D1 (PsbA) and D2 (PsbD) forming the core heterodimer; the chlorophyll-*a* binding core antennas CP43

(PsbC) and CP47 (PsbB); the α and β subunits of cytochrome-b_{559} (PsbE and PsbF respectively); and the low molecular weight proteins PsbH, PsbI, PsbJ, PsbK, PsbL, PsbM, PsbN and PsbX.

Gene / protein	Subunit (Common name) / topology	Gene location	Found	Prot.size (kDa)/ transmemb. Helices
psbA / PsbA	D1-intrinsic	c	all	38(S) / 5
psbB/ PsbB	CP47- intrinsic	c	all	56(S) / 6
psbC / PsbC	CP43- intrinsic	c	all	50(S) / 6
pcbD / PsbD	D2 –intrinsic	c	all	39.4(S) / 5
psbE / PsbE	α-b559-intrin.	c	all	9.2(S) / 1
psbF / PsbF	β-b559 - intrin	c	all	4.4(S) / 1
psbG / PsbG				7.7 (S) /1
psbH /PsbH	10kDa phosphoprotein – intrinsic	c	all	9 (Sy) /1
psbI / PsbI	I – intrinsic	c	all	4 (P) /1
psbJ / PsbJ	J – intrinsic	c	all	4 (S) / 1
psbK / Psb K	K - intrinsic	c	all	4.3 (S) / 1
psbL / Psb L	L – intrinsic	c		
psbM / PsbM	M – intrinsic	c		
psbN / PsbN	N – extrinsic	c		
psbO /PsbO	OEE1, 33kDa– lumenal	n		
npsbP /PsbP	OEE2, 23kDa	n	green algae	
psbQ /PsbQ	OEE3, 16kDa	n	green algae	
psbR /PsbR		n	green algae	
psbS /PsbS		n	green algae	
psbT1 /$PsbT_c$	ycf8 - intrinsic	c	all	
psbT2 /$PsbT_n$*	Lumenal	n	green algae	
psbU /PsbU**	lumenal	n	cyanobacteria and non-green algae	
psbV /PsbV**	Cyt c_{550} lumenal	c	cyanobacteria and non-green algae	
psbW /PsbW*		n	green algae	
psbX /PsbX		n	all	
psbY /PsbY	ycf32	n	all	
psbZ /Psbz	ycf9	c	all	

Table I.2 **: Photosystem II genes and subunits in plants, algae and cyanobacteria.**Table show the protein subunits that constitute the core of PS-II. These proteins are found in all oxygen evolving photosynthetic organisms. Those found exclusively in plants and algae are marked with an asterisk (*) and those in cyanobacteria with (**). In eukaryotes, genes are located in either the chloroplast (c) or the nuclear (n). The molecular masses correspond to mature proteins and are calculated from the protein sequences reported in the SWISSPROT database using the MacBioSpec (Sciex Corp., Thornhill, Ontario, Canada) for spinach (S), pea (P), Tobacco (T) and *Synechococcus sp* (Sy). The number of predicted transmembrane helices is based on hydropathy analyses of primary sequence.

The backbone of PS-II reaction centre is a heterodimer complex constituted of D1 and D2 proteins, which together bind all the cofactors involved in the light-driven electron transport reactions. Both subunits contain five membrane-spanning *a*-helices with their N-termini exposed to the stromal surface of the membrane. The two subunits are related by a pseudo-two-fold symmetry axis, named pseudo-C2 axis, which is oriented perpendicular to the thylakoid membrane plane and passes through the non-heme iron located at the stromal side of the membrane. Along with the intimately associated D1 and D2 proteins, the two core light-harvesting antennas, known as CP43 and CP47, and the α (PsbE) and β (PsbF) subunits of cytochrome-b_{559} constitute the main intrinsic subunits of PS-II reaction centre. The complex of the above mentioned polypeptides along with the OEC and the extrinsic PsbO subunit is capable of oxygen evolution at high rates and is known as BBY complex named after Berthold, Babcock & Yocu who first reported it (Berthold *et al.,* 1981).

The two homologous Chl-*a* binding proteins, CP43 and CP47 flank the sides of the heterodimer D1/D2. Both inner-antenna subunits (CP43 & CP47) consist of six transmembrane α-helices arranged as a trimer of dimmers related by the same pseudo-C2 axis relating the D1 and D2 subunits. The two-fold symmetry breaks with the cyt-b_{559}. Taken together, all the intrinsic proteins constituting the PS-II core complex, give rise to at least thirty six transmembrane α-helices (Zouni *et al.,* 2001; Guskov *et al.,* 2009) twenty-two of which are assigned to D1, D2, CP43 and CP47. The two subunits of cyt-c_{559} are proteins of low molecular weight and each span the thylakoid membrane as a single α-helix. The small subunits, PsbH, PsbI, PsbJ, PsbK, PsbL, PsbM, PsbN and PsbX are forming a single transmembrane α-helix and none of them have redox active compounds.The functional role of most of these proteins is not known yet, since mutants in which many of these proteins have been deleted appear to grow as well as the wild-type organisms.

The photosystem-II RC D1 and D2 proteins.

The PsbA and PsbD core subunits are commonly known as D1 and D2 proteins. The nomenclature was first introduced during the research for identification of chloroplast-encoded proteins in *Chlamydomonas reinhardtii* (Chua & Gillham, 1977). In pulse chase studies with [^{14}C]-acetate in the presence of an inhibitor of cytoplasmic protein

synthesis autoradiography revealed two broad areas of radioactivity termed 'Diffuse band-1' and 'Diffuse band-2' – hence the common names D1 and D2 respectively.

In analogy to L and M subunits of the purple bacterial RC, D1 and D2 proteins together bind all the cofactors of the light-driven electron transfer reactions. Despite the low sequence similarity (~15%) to L and M subunits of PbRC, D1 & D2 proteins are regarded as homologous to L & M, mainly because of the highly conserved sequences corresponding to the binding regions of photochemically active cofactors (Michel & Deisenhofer, 1988).

D1 subunit

D1 polypeptide has highly conserved amino acid sequence and undergoes posttranslational, N and C termini modifications before reaching its mature form as core subunit. At this stage it has a molecular mass of about 38kDa, and forms five membrane-spanning α-helices (I to V) and two surface helices between III and IV (lumenal) and IV and V (stromal)

The D1 subunit is characterised by some distinctive features. It binds the majority of the cofactors involved in the electron transfer system: P_{D1} (possibly via His 198); Y_Z (tyr-161) that bridges the WOC and the P_{680}; Chl_{D1}; the early electron acceptor $Pheo_{D1}$; the non heme iron (probably via His 215 &272); the quinone Q_B; and the manganese cluster. The unique feature of D1 is that it turns over more rapidly than any other protein involved in photosynthesis (Mattoo *et al.,* 1984). It is generally believed that this characteristic of D1 to be damaged and replaced so frequently is mainly attributable to unavoidable side oxidation reactions that occur despite the operation of protective mechanisms. The damage of D1 protein consequently leads to reduction of photosynthetic activity, but this particular matter (photoinhibition) is discussed in more details below.

D2 subunit

D2 polypeptide similarly to D1, produces five membrane-spanning helices and binds the rest of the cofactors of the ETS: P_{D2}; photochemically inactive $Pheo_{D2}$; Chl_{D2} and the electron stabilising quinone Q_A. It is worth mentioning that under extreme conditions D2 also turns over at measurable rates.

Light-harvesting core antenna: CP43 and CP47 subunits.

The D1 & D2 heterodimer is complemented by an outer core antenna of two Chl-*a* binding proteins CP43 and CP47. These core antennas are structurally independent from the outer light harvesting complex (LHC-II) in higher plants and green algae and from PBS in cyanobacteria.

These two Chl-*α* binding proteins are the largest subunits of photosystem-II and each spans the lipid bilayer with six transmembrane *α*-helices. The sequence similarity between the PS-II core antennas CP43 & CP47 (transmembrane segments I & IV) and the N-terminal antenna-binding domains of the core proteins of type-I RC, PsA, PsaB, PsacA and PshA (of PS-I, PS-C and PS-H respectively) (Fromme *et al.,* 1996) – supports the hypothesis that all RC share the same evolutionary origin. However, in contrast to all known type-I RC, which combine a RC and an antenna domain in a single protein subunit, the PS-II core antennas CP43, CP47 are distinct from the core subunits D1 & D2. Apart from D1, D2, CP43 and CP47, none of the PS-II subunits has been shown to bind Chl-*a* of the core antenna or electron transfer system.

PsbB (CP47) subunit is highly conserved among oxygenic photosynthetic organisms and forms six transmembrane *α*-helices, with the N and C termini exposed to the stromal surface (Bricker 1990). It also contains a large lumenal loop (~200 amino acids) between V and VI transmembrane domains.

CP47 binds a number of cofactors including chlorophyll-*α* and *β*-carotenes but does not bind Chl-*b* or pheophytins (Alonso *et al.,* 1994). However, the number of chlorophylls bound to CP47 is still an issue under discussion and it appears to be in the range of 12-25 molecules (Alonso *et al.,* 1994; Barbato *et al.,* 1991). The prime candidate for chlorophyll binding, seems to be the histidine residues. Also, the amino acids glutamine and glycine, as well as histidine have been identified to act as chlorophyll ligands in LHC-II (Kuhlbrandt *et al.,* 1994). The structural model of PS-II obtained at 3.8Å resolution (Zouni *et al.,* 2001) suggests that there are fourteen chlorophyll and three *b*-carotene molecules bound to CP47, which are arranged in two layers; close to the stromal and lumenal side of the membrane respectively. These data are in contest with the observation that there are fourteen conserved histidines residues, of which twelve are located within the transmembrane helices and are also arranged in two layers.

The distance between some of the Chl-*α* molecules within the protein is close enough to propose, that they may be excitonically linked to one another. The latter in turn correlates with the energy transfer function of CP47. However, due to large spatial separation of both core antennas (CP43, CP47) from P_{D1} and P_{D2} direct exciton transfer from the antennas to the primary donor P_{680} seem to be forbidable and a possible route for the energy transfer may include the combinations ChlzD1/Chl_{D1} and ChlzD2/Chl_{D2}.

A wide range of site-directed mutational studies indicates that CP47 subunit, apart from its prime function of excitation energy transfer, plays essential role in the assembly and function of PS-II, and also consists a prerequisite for photoautotrophic growth of the cyanobacterium *Synechocystis* 6803.(Vermaas *et al.,* 1986; 1988). The involvement of CP47 in excitation energy transfer and its interaction (via the lumenal loop between V and VI transmembrane helices) with the manganese stabilising protein (PsbO) explains why deletion of *psb*B gene inhibits the assembly and stability of PS-II (Odom & Bricker 1992; Putnam-Evans & Bricker 1992; Vermaas *et al.,* 1986; 1987)

PsbC (CP43) subunit is homologous to CP47 and both proteins share a great number of structural and functional similarities. Like its counterpart, CP43 contain six transmembrane *α*-helices, one large lumenal loop (~150 amino acids) between the V and VI hydrophobic domains and similarly exposes its N and C termini to the stroma (Bricker 1990). The proposed number of bound chlorophyll-*α* molecules vary, ranging between 9-25 chlorophylls per subunit (Alonso *et al.*, 1994; Barbato *et al.,* 1991). The arrangement of twelve histidine residues in two layers within the membrane, exhibits remarkable similarity to that in CP47.

CP43, alike its counterpart CP47, performs light-harvesting and energy transfer. Mutational studies have revealed that deletion of *psb*C gene seriously affects the assembly of PS-II and the water oxidation (Roegner *et al.,* 1991) although the impact is not as severe as the deletion of *psb*B gene. Again, the lumenally exposed loops and most importantly the loop between the IV and V transmembrane domains seem to be partially involved in providing the right environment for the manganese cluster. Indeed, any mutations in this loop result in loss of oxygen evolution or altered S-state cycling (Carpenter *et al.,* 1993).

Regardless of any similarities between CP43 and CP47, there are two important differences: in higher plants only, the threonine residue at the N-terminus can be irreversibly phosphorylated (Michel & Deisenhofer 1988); CP43 protein can be easily removed from the photosystem-II RC yielding a CP47-RC complex (Ghanotakis *et al.,* 1989; Dekker *et al.,* 1990). This particular characteristic feature of CP43 subunit seems to be of great importance for the repair cycle of D1 protein.

Cytochrome-b_{559}. Structure and function.

The cytochrome-b_{559} is a heme protein, which consists of two subunits referred to as α (Psb E) and β (PsbF) subunits. Both polypeptides contain a single membrane-spanning α-helix and a stromally exposed N-terminus. The latter contains a methionine residue which is removed after the translation, yet the same side of β subunit only undergoes additional acetylation. The heme group is attached to the to polypeptides via a single histidine residue present in their sequence and located within the hydrophobic domains. The two subunits cross-linked by the heme group form a heterodimeric complex.

Despite the extensive research in last few decades, there are still two main, not fully elucidated issues over the structure and function of cytochrome-b_{559}. The first, is how many cytochrome-b_{559} dimers (one or two) exist in a reaction centre. Although most of the data indicate the presence of a single cytochrome per RC, this may depend on the preparation or / and the organism.

The second subject of discussion has to do with the functional role of cytochrome b-559. Even though several different functions have been postulated for this subunit, the most favoured one is its involvement in the photoprotective mechanism against the light-induced damage of the reaction centre.

Any disruption of normal electron flow through PS-II, inevitably leads to production of reactive species with detrimental effects on the RC. The general consensus is that the main causes of PS-II damage are the photoinduced highly reactive species of P_{680}^{+} and singlet oxygen ($^1O_2$). The oxidised primary donor, due to its remarkable oxidising potential and increased lifetime, leads to irreversible oxidation of carotenoids, chlorophylls, and the amino acids closely associated with it. This happens when the electron flow from the WOC towards P_{680} via Y_Z is either disrupted or insufficient. The production of the highly reactive and toxic singlet oxygen involves the triplet P_{680},

which is formed by recombination of $P_{680}^{+}Pheo^{-}$ when Q_A is doubly reduced. The role of cytochrome-b_{559} in the photoprotective mechanism relies on its ability to act either as electron donor or electron acceptor. It has been reported that this cytochrome exists in high or low potential. The high potential form of cyt-b_{559} donates electrons to P_{680}^{+} thus providing protection from harmful, non-specific oxidising reactions of the radical cation. In contrast, the low potential form of cyt-b_{559} act as an electron acceptor and by doing so it oxidises $Pheo^{-}$, thus preventing the recombination in the radical pair $P680^{+}$ $Pheo^{-}$**,** and the consequential formation of triplet oxygen.

While there is little or no doubt at all about the ability of cyt-b_{559} to act as an electron donor or acceptor, the pathway of these reactions is still a matter of intense discussion. It is not clear yet, whether electrons go from cyt-b_{559} to P_{680} via ChlzD2 or directly via β-carotene.

Apart from its involvement in the photoprotective mechanism, cyt-b_{559} and in particular the histidine residues in the transmembrane domains of α and β subunits seem to play an important role in maintaining the structural integrity of PS-II. In cyanobacterium *Synechocystis* 6803, site directed mutant (histidine residues in both subunits), as well as *psb*E and *psb*F deletions mutants all fail to accumulate D1 and D2 proteins (Pakrasi *et al.,* 1990; 1991).

Low molecular weight subunits.

All small subunits of photosystem-II (exception the Lhc-like PsbS protein) have molecular masses under 10kDa. The exact functional role of these proteins is not yet clear, as deletion mutants appear to grow nearly as well as the wild type organisms. None of the small subunits associated with PS-II reaction centres has been reported to bind redox active centres.

PsbH subunit.

This protein is present in all oxygen evolving photosynthetic organisms, and it contains a single transmembrane domain located towards its C-terminus. The lumenally exposed N-terminus can undergo reversible phosphorylation in higher plants (Michael and Bennett 1987). The phosphorylation occurs when the PQ pool is fully reduced and it appears to play a role in stabilisation of Q^{-}_{A} (Packham 1988). In cyanobacteria (*Synechocystis* 6803) PsbH-less mutants show modified Q_A to Q_B electron transfer and

are prone to photoinhibition, mainly due to impaired repair cycle rather than to increased photochemical damage (Mayes *et al.,* 1993; Komenda & Barber 1995)

PsbK subunit.

The PsbK subunit is an intrinsic protein present in all oxygen evolving photosynthetic organisms but its functional role is not yet clear as deletion mutants in cyanobacteria and green algae show different phenotypes. While PsbK-less mutants of *Synechocystis* 6803 are able to grow photoautrophically and exhibit no enhanced susceptibility to photoinhibition (Ikeuchi *et al.,* 1991; Zhang *et al.,* 1993); in green alga *Chlamydomonas* deletion of the same gene prevented photoautrophic growth and the mutants accumulated fraction of wild type PS-II levels (Takahashi *et al.,* 1994). The results suggest that in *Synechocystis* 6803 the PsbK protein may play a role outside of the normal functioning of PS-II, while in green alga, the PsbK subunit obviously appears to be important for the assembly or stability of the photosystem-II complex.

PsbL subunit

The PsbL subunit is an intrinsic, single transmembrane polypeptide found in all oxygen evolving phototrophs. This protein seems to be important for normal functioning of the Q_A site since its removal from isolated photosystem-II core complexes dramatically decreases Q_A functioning (Kitamura *et al.,* 1994). This small protein in cyanobacteria seems also to be involved in the oxidation of Y_Z by P_{680}^+ (Hoshida *et al.,* 1997).

PsbX subunit.

Deletion of *psb*X in cyanobacteria seems to reduce the level of photosystem-II and therefore this particular subunit may be involved in regulation of the amounts of functional PS-II (Funk , 2000)

6.2.2. Photosystem-I (PS-I).

Introduction

Photosystem-I is a large multi-subunit protein complex embedded into the TM of oxygenic photosynthetic organisms. Working in series with PS-II, it catalyses the electron transfer from plastocyanin or cytochrome-c_6 on the lumenal side of the membrane to ferrodoxin or flavodoxin on the stromal side of the membrane that eventually reduces NADP+ to NADPH via ferrodoxin-NADP-oxireductase (FNR). Unlike photosystem-II, which operates in a highly oxidising regime, PS-I is mach more

reducing (the excited state P_{700}* of the primary donor is estimated to be –1.26V) (Chitnis, 1996; 2001). In an alternative pathway, the soluble electron carrier ferrodoxin re-reduces plastocyanin via cyt-b_6f complex. This cyclic electron flow around PS-I RC, which does not require the input of free energy from PS-II, increases the electrochemical potential across the membrane required for ATP production. The light generated reducing power of NADPH, along with the energy carried in ATP, are subsequently used to reduce CO_2 to carbohydrates in series of enzymatic reactions.

Structure and composition

The basic structures of PS-I reaction centres in cyanobacteria, green algae, and higher plants are remarkably similar. The core polypeptides providing the scaffold for the redox active centres, seems likely to be identical in PS-I of all oxygen evolving organisms.

The structural model of PS-I obtained at 2.5Å (Jordan *et al.,* 2001) provides great insights into the molecular architecture of this complex. The structure has analogies to that of PbRC, as the more emphatic is the organisation of the cofactors of the ETS. However there are differences in the overall structure, since PS-I is more complex and there are over 14 distinct subunits associated with this RC. Apart from the constituent subunits, the reaction centre alone (not including the LHC-I) binds and coordinates a total of 127 cofactors (Jordan *et al.,* 2001): 96 chl-*α*, 22 carotenoiods, two phylloquinones, three iron-sulfur clusters and four lipids (Jordan *et al.,* 2001). Table I.3 summarises the constituent polypeptides found in PS-I RC of all oxygenic photosynthetic organisms including their location and the cofactors they are associated with.

The core of the reaction centre consists of a heterodimer of two membrane intrinsic subunits, PsaA and PsaB. These two major polypeptides are related by a local pseudo-C2 axis of symmetry. They bind the majority of the cofactors of the ETS, from the primary donor P_{700} up to the first iron-sulfur cluster (F_X). This makes up a total of six Chl-*α*, two phylloquinones (A1) and the F_Z (Jordan *et al.,* 2001). The other two iron-sulfur clusters (F_A, F_B) involved in the electron transfer chain are coordinated and bound to an extrinsic, stromaly exposed 8kDa protein known as PsaC subunit.

The PsaA and PsaB, although show marked resemblance to the core polypeptides of either PbRC or PS-II, are characterised by some unique features. Along with their high molecular weights (of about 83kDa each), is the organisation of the associated cofactors and the light harvesting chlorophylls into two distinguishable domains within the thylakoid membrane that makes these proteins so unique. Booth subunits contain 11 transmembrane α-helices (A/Ba to A/Bk) and expose their N-termini to the stroma whereas the C-termini are located in the lumenal side of the membrane. While the local pseudo two-fold symmetry, which relates the two subunits is well preserved in the membrane integral parts; it notably breaks in loop regions. The five C-terminal α-helices (PsaA, PsaB) are referred to as the reaction centre domain because these helices bind the majority of the cofactors of the ETS, and structurally separate them from the antenna pigments of PS-I. These transmembrane domains exhibit significant similarities to L/M of PbRC and D1/D2 of PS-II, but unlike them, the C-terminal domains of PsaA and PsaB in addition to the bound cofactors also coordinate 25 chlorophylls (12 in PsaA and 13 PsaB) of the antenna system. At the stromal side of the membrane, the remaining α-helices of PsaA and PsaB coordinate 23 additional Chl-α molecules. A unique feature of PS-I core subunits is the fact that the chlorophylls are not exclusively coordinated by the amino acids residues in the transmembrane helices, but instead, the 31 additional Chl-α molecules are bound by amino acids side chains located in the loop regions. All these 54 molecules of chlorophyll located at the stromal side of the membrane form the so-called antenna domain. Yet, the high resolution (2.5Å) structure confirms the presence of four lipids in PS-I, located in the core of PsaA and PsaB at positions following the local two-fold pseudo symmetry.

Electron microscopy studies (Jekow *et al.,* 1996) of isolated gross PS-I complexes show that *in vivo* the cyanobacterial photosystem-I occurs as a trimer, whereas the eukaryotic PS-I exists as a monomer. Mutational studies indicate that PsaL subunit consists a prerequisite for the formation of trimers in cyanobacteria (Jekow *et al.,* 1996). While this protein is found in all oxygenic photosynthetic organisms, it seems that in higher plants and green algae it does not promote the formation of trimers even though it is essential for PS-I assembly. In higher plants and green algae the PS-I complex is composed of two structurally and functionally distinct parts: the RC and the Light-Harvesting complex-I, (LHC-I).

Gene/subunit / location of the gene	Found	Mass (kDa) / Topology	Cofactors	Function
psaA / PsaA/c	all	84/TM11	96 Chl-α, 22 β-carotenes, P700, A_0, F_X.	Light harvesting, elect.transport
psaB / PsaB /c	all	83/TM11		
psaC / PsaC /c	all	9/ extr.stromal	F_A, F_B	electron transp.
psaD / PsaD /n	all	18/ extr.stromal	-	Ferrodoxin docking
psaE / PsaE /n	all	10/extr. stromal	-	cyclic elect.transport, binding PsaC, binding ferrodoxin and FNR
psaF / PsaF /n	all	17/TM1	carotenoid cluster (5)-	plastocyanin docking, binding LHCI-730
psaG / PsaG /n	eukaryotes	11/TM2	-	binding of LHCI-680
psaH / PsaH /n	eukaryotes	11/extr. stromal	-	binding og LHCII-state transitions. Stabilization of PSI - D
psaI / PsaI /c*	all	4/TM1	carotenoid cluster (6)	stabilization of PSI-L
psaJ / PsaJ /c	all	5/TM1	Antenna chlorophylls: 3	stabilization of PSI-F
psaK / PsaK /n	all	9/TM1	antenna chlorophylls: 2, carotenoids cluster 1	binding of LHCI-680
psaL / PsaL /n	all	18/TM2	antenna chlorophylls: 3	stabilization of PSI-H
psaM / PsaM/c	cyano.	3/TM1	antenna chlorophylls: 1 carotenoid cluster: 6	
psaN / PsaN/n	eukaryotes	10/extr. lumenal	-	docking of plastocyanin
psaO / PsaO/n	green algae	9/TM2		unknown
psaX / PsaX	cyano.	6/TM1	antenna chlorophyll: 1	uknown
lhca1/LHCI-720 /n	eukaryotes	22	~10 Chl-α, ~2 Chl-b, ~3 carotenoids	Light harvesting, LHC-I-730
lhca2/LHCI-680 /n	eukaryotes	23	~10 Chl-α, ~2 Chl-b, ~3 carotenoids	Light harvesting LHC-I-680B
lhca3/LHCI-680 /n	eukaryotes	25	~10 Chl-α, ~2 Chl-b, ~3 carotenoids	Light harvesting LHC-I-680A
lhca4/LHCI-720 /n	eukaryotes	21	~10 Chl-α, ~2 Chl-b, ~3 carotenoids	Light harvesting LHC-I-730

Table I.3. **Subunits of PSI complex.** The location of genes in higher plants and green algae: c - in chloroplasts, n - nucleus. For core proteins, the number of transmembrane domains (TM). PsaI is nuclear encoded in some green algae such as *Chlamydomonas*.

Electron transfer within the Reaction Centre.

The overall process of energy conversion by light-induced electron transfer leading to charge separation across the membrane, is literally the same in all photosynthetic reaction centres. Intensive spectroscopical studies in the last few decades over the kinetics of the electron transport system showed that the electron transfer pathway is accomplished by a chain of redox active centres arranged in the following order: P_{700} – the primary donor (probably a dimer of Chl-*α*); A_0 – a Chl-*α* molecule; A_1 – a phylloquinone, also known as vitamin K1; and three iron-sulfur clusters designated as F_X, F_A, and F_B. The relatively high-resolution structure of PS-I (Jordan *et al.,* 2001) has provided great insights into the structural details of the cofactors and the protein-cofactors interactions, which define the kinetics of the electron transfer reactions. At resolution better than 3Å, the X-ray diffraction densities can be fitted to amino acids in polypeptide sequence, thus allowing the allocation of roles to precise amino acids. The new high-resolution (at 2.5Å) model of PS-I simply confirms the spectroscopically established set of ETS cofactors.

Although the entire pathway transfers a single electron, gating to a two-electron transfer at FNR, the intermediates of the ETS are arranged into two, nearly symmetrically branches creating two potential electron pathways in the reaction centre (Figure I.4).

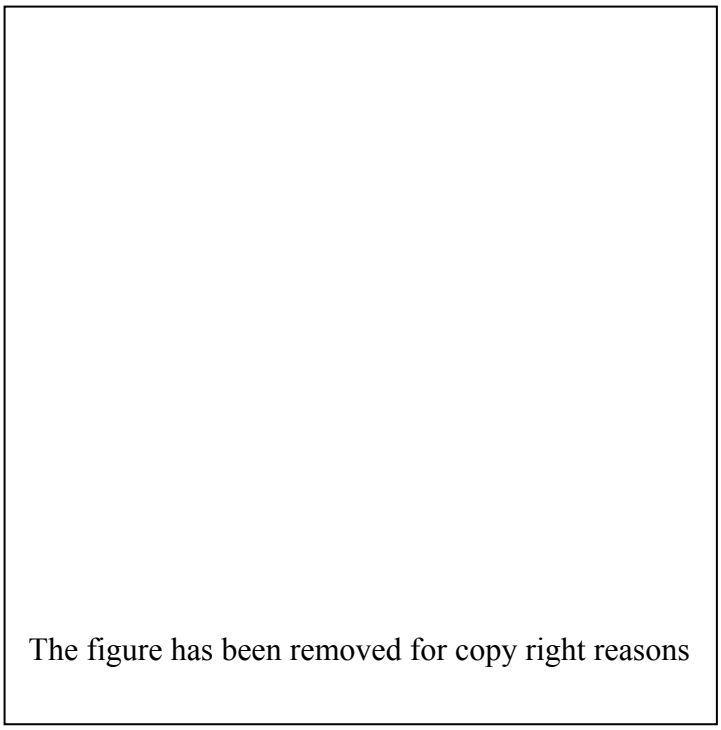


Figure I.4: **The electron transfer cofactors in Photosystem I.** The A^- and B^- side cofactors are related by C_2 symmetry. There are potentially two routes for electron transfer from P_{700} to F_X. Reproduced from: http://www.queenmaryphotosynthesis.org/krauss/photosystems/page18/files/pasted-graphic-1.jpg

The general arrangement of the chlorophyll and quinone cofactors of the ETS in PS-I is very similar to that in type-II reaction centres. Nevertheless, the electron transfer chain in PS-I is more extensive than in type-II centres because of the three iron-sulfur clusters at the stromal side of the membrane.

Electron transfer towards and from PS-I

The electron transfer pathway within the cyanobacterial PS-I reaction centre is the same as that in green algae and higher plants. However, there is a major difference in the overall electron transfer process, and it is associated with the nature and variability of the soluble electron carriers involved in the process as electron donors and acceptors at lumenal and stromal side of the membrane respectively (Raven *et al.,* 1999).

Supply of electrons from PS-II to PS-I via cyt-b_6f complex requires the presence of free diffusible electron carriers in the lumen of the thylakoid. In higher plants this task is accomplished by a small copper protein known as plastocyanin (PC) which shuttles electrons from cyt-b_6f to PS-I. In contrast, in cyanobacteria and most of green algae a *c*-

type cytochrome, called cytochrome c_6 is found either in addition to or instead of plastocyanin (Kerfeld, 1997). The relative amounts of the two proteins present in the lumen are controlled by the availability of Cu in the growth media (Merchant & Dreyfuss, 1998). Deletion of both genes (PC and c_6) in *Synechocystis* inhibits completely the photoautrophic growth (Manna & Vermaas, 1997).

The final and most frequent destination in the non-cyclic electron transfer chain is the reduction of NADP+ mediated by ferredoxin-$NADP^+$-oxireductase (FNR). This step also requires freely diffusible electron carriers on the stromal side of the reaction centre. In cyanobacteria and some green algae, this function is performed by a small soluble electron carrier protein called ferredoxin (Fd), which under stress conditions (Fe starvation or high salt) is replaced by flavodoxin (Fld)(Hagemann et al., 1999). In higher plants it has been thought that ferredoxin is the exclusive electron transfer protein from PS-I, even though flavodoxin encoding genes have also been identified in *Arabidopsis thaliana*. Despite the presence of such genes it is still unclear whether flavodoxin can replace ferredoxin in eukaryotes *in vivo*.

As mentioned above, the final phase in linear electron transfer chain is the reduction of $NADP^+$ to NADPH. The process is mediated by FNR and requires the addition of two electrons and one proton. Ferredoxin and Fld are both a single electron carriers. FNR thus converts the intrinsically "one electron at a time chemistry" of the RC into "two electrons at a time chemistry", which is characteristic of most chemical reactions. In addition to the major role in reducing $NADP^+$ via FNR, ferredoxin (or / and flavodoxin) also functions as an electron donors to a number of soluble enzymes involved in nitrogen & sulfur metabolisms and in the regulation of carbon metabolism (Knaff, 1996). Furthermore, Fd can be involved in an oxygen removing mechanism from PS-I; a process known as Mehler reactions. Reduced Fd is readily oxidised by dioxygen forming superoxide radicals (O_2^-), which are subsequently catalysed by the enzyme superoxide dismutase (SOD) to form hydrogen peroxide (H_2O_2). The hydrogen peroxide can be then converted into water by ascorbate peroxidase.

Cyclic electron transfer around PS-I

The reduced Fd can catalyse a cyclic electron flow around PS-I reaction centre, where electrons re-reduce the oxidised primary donor ($P700^+$) via cyt-b_6f complex (Arnon 1984; Fork & Herbert, 1993). Although, ferredoxin-catalysed cyclic electron flow

around PS-I is easily activated by breaking the chain of electron transfer from PS-II (e.g. using inhibitors as DCMU), its physiological importance in intact photosynthetic organisms is still a matter of dispute. The cyclic paths of electron transfer around PS-I have been suggested to protect PS-II from photoinhibition (Heber & Walker 1992; Herbert *et al.,* 1995, Endo *et al.,* 1999).

The most familiar cyclic electron flow around PS-I involves a putative ferredoxin-plastoquinone oxireductase (FQR) that transfers electrons from Fd to PQ (Scheller 1996; Endo *et al.,* 1997). This cycle is completed when electrons from reduced PQ return to PS-I via cyt-b_6f complex.

A longer PS-I cycle involving a thylakoid NAD(P)H-plastoquinone oxyreductase (NDH-1) is also known (Yu *et al.,* 1993; Shikanai *et al.,* 1998). In this cycle, NADPH (generated by the linear electron transfer reactions) is oxidised and PQ is reduced by NDH-1. From PQ onwards, the pathway is the same as in the FQR cycle. In cyanobacteria, the thylakoid NDH-1 is also involved in respiratory electron transport as well as in the PS-I cycle (Peschek 1996).

How the cyclic electron transfer around PS-I may protect against photoinhibition is still unclear and several hypotheses have been postulated. In higher plants and green algae the PS-I cycles sustain a proton gradient when PS-II and non-cyclic electron transfer are inhibited.

In cyanobacteria, state transitions are very common and are dependent on the operation of PS-I cyclic electron flow (Hebert *et al.,* 1992). Redistribution of excess of energy between PS-II and PS-I during state transitions may be photoprotective since, at normal temperatures PS-I is less susceptible to photooxidative damage than PS-II (Martin *et al.,* 1997). However, the significance of state transition in the overall photoprotection mechanism is not yet clear. It has been shown (Emlyn-Jones *et al.,* 1999; Mullineaux & Emlyn-Jones 2004) that state transitions are required and carried out under low light only, and with an exclusive purpose, to maximise the efficiency of light harvesting.

In an indirect way the PS-I cyclic electron flow may also contribute to the repair mechanism of photodamaged PS-II, by increasing the ratio of ATP to NADPH that is generated by the linear electron transport chain. Synthesis of D1 polypeptide requires

approximately 350 mol ATP per mol protein. Unlike eukaryotes that can gain part of the ATP from cytosolic and mitochondrial sources, cyanobacteria that lack these external sources of ATP, PS-I driven cyclic phosphorylation may provide the additional ATP needed for the repair mechanism and the *de novo* protein synthesis. Yet, the ATP derived from cyclic electron flow is used to drive active uptake of CO_2 (Okhawa et al., 2000a; 2000b), which in turn can protect against photoinhibition.

6.3. Thylakoid membranes, structure, and dynamics

The light driven reactions of photosynthesis take place in uniquely specialised for the purpose membranes known as Thylakoid Membranes (TM). They are dynamic systems and subject to adaptation under continuously changing conditions. Thylakoid membranes are vesicular systems and enclose a lumenal space (thylakoid), which is sealed off from the cytoplasm to allow generation of chemiosmotic potential between the lumenal and stromal side of the membrane.

The photosynthetic membranes of oxygenic photosynthetic organisms have been extensively studied over the last several decades. The thylakoid membranes in chloroplasts are organised into two compartements; the grana (stacked) and stroma (unstacked) lamellae, as the latter interconnects the grana stacks (Anderson & Anderson 1980).

Electron microscopy in combination with freeze-fracturing technique of the specimen have revealed that the spatial distribution of membrane protein complexes in chloroplasts, is neither random nor homogeneous. The thylakoid membranes of chloroplasts show remarkable 'lateral heterogeneity' (Anderson & Melis 1983; Anderson & Anderson 1988; Anderson 2002, Dekker & Boekema 2005). In particular, PS-I and ATP synthase are found mainly in unappressed (stroma lamellae) regions, while a large portion of PS-II and LHC-II are localised in appressed grana. The distribution of cytochrome-b_6f complex is however under discussion as there are models proposing different patterns for its distribution between grana and stroma lamellae (Dekker &Boekema 2005). Some suggest almost equal distribution of the complex between the grana and sroma, while others propose a reduced number or even complete absence of cyt-b_6f complex from grana.

As PS-II centres exist in two different structural, and perhaps functional, forms, namely dimeric and monomeric, distribution of these two distinct forms within the thylakoid

membranes merits some special attention. The dimeric super-complexes of PS-II are found mainly in the grana while the monomeric forms in stroma. In contrast to granal PS-II, those found in stroma are not active in electron transport to PQ and also have limited antenna size (Anderson & Melis 1983). Identification of different types of photosystem-II gave rise to various interpretations. However, the most plausible explanation is related to PS-II turnover, but whether the inactive stromal PS-II forms are photodamaged photosystems underwent repair or are newly synthesised ones is not yet fully elucidated.

In cyanobacteria, TM constitute an extensive intracellular membrane system that often occupies the largest part of the cytoplasm. The extent of interconnection between thylakoid membranes as well as thylakoid-cytoplasm membranes is not clear yet. In contrast to chloroplast, thylakoid membranes in cyanobacteria do not form membrane stacking. Instead, they are aligned concentrically around the periphery of the cell (Sherman *et al.,* 1994). Species epitomising in the best observable way this pattern are those with elongated cells such as those from genus *Synechococcus*.

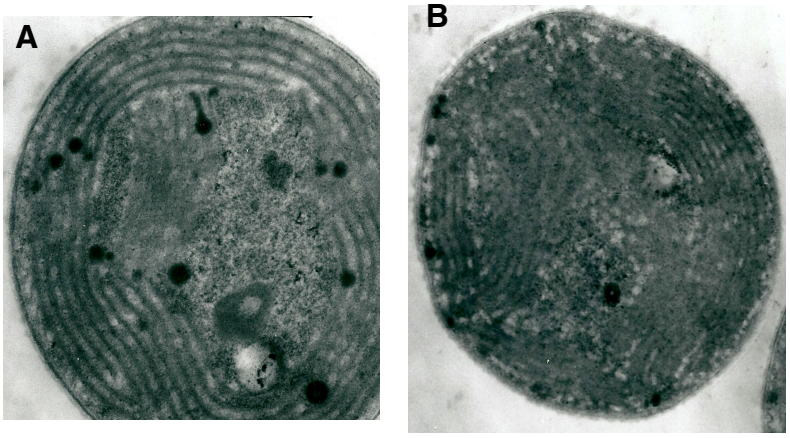


Figure I.5. **Transmission Electron Microscopy (TEM) images from ultra thin sections through photo-autotrophically grown *Synechocystis* 6803 cells.** Arrangement of thylakoid membranes in concentric cylinders around the periphery of the cell. A) Wild Type *Synechocystis* 6803 cell grown under normal incubatory conditions (30^{o} C and 10 μmol. m-2.s-1 irradiance). B) *Synechocystis* 6803 cells lacking FtsH protease (slr0228^{-} mutant) grown under the same laboratory conditions. Magnification for both images x50,000. TEM Images were obtained in order to understand whether the disruption of *slr*0228FtsH protease had affected the distribution of TM.

Along with the protein complexes of the electron transport system and the ATP synthase, the cyanobacterial thylakoid membranes, in contrast to chloroplasts, contain also respiratory enzymes including and NADP(H)-plastoquinone oxireductase (Berger *et al.,* 1991). The amount of cytochrome oxidase in thylakoid membranes varies amongst cyanoabacteria. In *Synechocystis* 6803 and *Cyanothece* for example, the cytochrome oxidase resides mainly in TM whereas in *Synechococcus* 7942 the same enzyme is almost exclusively found in plasma membrane (Sherman *et al.,* 1994). Both, *Synechocystis* and *Cyanothece*, are species capable of photoheterotrophic and heterotrophic growth. In contrast, *Synechococcus* 7942 is an obligate photoautotrophic organism. It appears thus that the presence of cytochrome oxidase, in either plasma or thylakoid membranes, relies primarily on the metabolic life style of the organism.

The distribution of photosynthetic protein complexes within cyanobacterial TM is relatively homogeneous, although radial asymmetry has been observed, with minor differences in the composition of inner and outer membranes. ATP synthase is localised mostly at the cell periphery, either in cytoplasmic or the outermost thylakoid membranes. Similarly to ATP synthase, photosystem-I is localised chiefly at the outer thylakoid membranes. In contrast, PS-II and cyt-b_6f complexes are distributed relatively evenly throughout thylakoids (Sherman *et al.,* 1994). Furthermore, freeze-fracture electron microscopy has revealed that the particles of PS-II dimmers are aligned in long, parallel rows (Morschel & Schatz 1987). The same arrangement, in parallel rows on the surface of the thylakoid membranes has also been observed with PBS, which (PBS) due to their size, are likely to determine the spacing between the PS-II rows (Olive *et al.,* 1997). However, alignment of PS-II dimmers in parallel lines among PBS containing organisms is not universally conserved, e.g. the red alga *Porphyridium cruentum* (Mustardy *et al.,* 1992).

Advances in electron microscopy (EM) and X-ray crystallography resulted in a tremendous progress in understanding the structure and organisation of thylakoid membranes and the distribution of protein complexes as well. Nevertheless, despite this remarkable progress, all of these methods tend to produce a rather static picture of the membranes and its components. Thylakoid membranes however, are dynamic systems and still little is known about the real time movement and interactions of its protein complexes. Utilisation of the natural fluorescence of thylakoid membranes and its

components in combination with fluorescence recovery after photobleaching (FRAP) have revealed some remarkable features of these dynamic systems. For instance, it has been shown that the light-harvesting phycobilisomes diffuse rapidly on the surface of the thylakoid membranes (Mullineaux *et al.,* 1997; Sarcina *et al.,* 2001), whereas the cyanobacterial PS-II reaction centres are surprisingly immobile, with no diffusion detectable even on long time scale (Sarcina & Mullineaux 2004). However, exposure of intact cells of *Synechococcus* 7942 to intense red light have led a significant proportion of PS-II as high as ~50% to diffuse relatively fast within thylakoid membranes and even to relocate to poles (Sarcina *et al.,* 2006). Yet, FRAP analysis has also shown that lack of particular FtsH from *Synechococcus* 7942 results in mobilisation of certain PS-II complexes even under normal conditions, i.e., without the red light pretreatment (for details see Chapter VI).

7. Photoinhibition.

Photoinhibition, which literally means light-induced inhibition of photosynthetic activity, is a phenomenon that occurs to all oxygenic photosynthetic organisms when exposed to light. So light, the ultimate driving force of photosynthesis, the source of almost all metabolic energy on this planet, can also be harmful for the photosynthetic apparatus.

In resent years, photoinhibition, very often, is defined as light-induced damage to PS-II, constricting thus the complexity of the phenomenon to the main site of the damage, namely the reaction centre of photosystem-II and more precisely to its D1 core sub-unit. Nevertheless, D1 is certainly not the only PS-II protein that is being subjected to light-induced irreversible damage and PS-II itself is not the exclusive site of damage. A number of proteins in both complexes, viz., PS-I and PS-II have been shown to undergo photodamage, impeding thus to one or another degree the photosynthetic performance of the organism (for details see below).

Although by definition in this process, light is the cause inhibiting the photosynthetic activity, it is without doubt, not the only one there is. Inhibition of photosynthesis occurs whenever the energy input to the reaction centres exceeds the capacity of the electron transport at this moment, which is contingent upon a multitude of factors, classified as time or duration of illumination, abiotic stress conditions and metabolic

needs of the organism at a given moment. All these factors, individually or collectively affect the balance of energy flow through photo-physical, and photochemical reactions to utilisation of the derived energy in biochemical metabolic reactions. Consequently, they determine the 'magnitude' of excitation pressure, thereby the extent of photoinhibition.

As the light-induced damage occurs at all light intensities and is irreversible, photosynthetic organisms have evolved a mechanism to restore the damaged units, and this mechanism is known as the repair mechanism or repair cycle. In these terms then, photoinhibition, or more precisely net photoinhibition occurs only when the rate of photo-induced inactivation of its complexes (PS-II or PS-I) exceeds the capacity of the repair mechanism at that moment at which the decline of photosynthetic performance becomes observable (for more details see below).

7.1. Historical and other aspects of photoinhibition

Photoinhibition, as a general phenomenon of decline of photosynthetic performance due to excess of light, has been known for over a century. In the later part of the 19th century it was established that the light, as any other substrate, could reach a level that saturates the photosynthesis and further increase of light intensity beyond the point of saturation lead to reduction of photosynthetic activity to the point of total inhibition.

Some of the earliest systematic studies of the phenomenon were published in 1896 by Alfred J. Ewart, who examined the ability of 'chlorophyll bodies' (chloroplasts) to produce oxygen in the presence of strong light. He detected this by the reduced numbers of oxygen requiring bacteria, congregated around photosynthetically active cells in leaf sections, indicating thus reduced oxygen evolution, i.e., in modern terms photoinhibition.

Despite these early interesting discoveries, the general view of photoinhibition, its underlying mechanism(s), the primary site of damage as well as other aspects of the phenomenon were unknown. Over the next decades, light saturation curves were measured for various photosynthetic organisms, very often as a function of a third parameter (pH, chemicals inhibiting photosynthesis etc). In the beginning of the 20th century, it was shown that light is not the only factor causing inhibition.

In an attempt to identify the nature of the primary photo-induced damage, it was proposed that the inhibiting effect of intense light is due to inherent heating of the chloroplast and leaf and not because of the photochemistry itself. Of course this idea turned out to be incorrect and a few decades later it was suggested that photoinhibition caused by either high levels of oxygen or high light intensity affects the catalytic mechanism of photosynthesis (reviewed in Barber & Anderson 1992; Prasil *et al.,* 1992; Aro *et al.,* 1993; Keren & Ohad 1998).

Progress in biophysics, biochemistry and other sciences, as well as in the available technology, improved the quality of analytical methods used in the study of photosynthesis. Research in the field of photoinhibition has changed from descriptive and physiological to its molecular aspects, from *in vivo* experimentation to the use of *in vitro* systems such as isolated thylakoid membranes or even sub-membrane particles.

Despite the accumulated knowledge on any level, from biochemical to *in vivo* systems, our understanding of the process is still far from complete and conclusive. Along with objective difficulties stemming from the complexity of the phenomenon itself, often, there may be subjective reasons due conventional research approaches. Thus it is often taken as granted, although a mere convention, that photosynthetic organisms must have evolved a sophisticated mechanism to deal with exposure to direct strong light, despite the fact that most of the chlorophyll on the planet does not operate under such conditions (found either in shade or in certain depth in water).

An undeniable difficulty is the complexity of the phenomenon that becomes even more intricate when considering the vast variability of photosynthetic organisms, adapted practically to almost all kind of environments. Thus, under certain conditions it is difficult to define whether the decline of photosynthetic activity, which we are referring to as photoinhibition, represents damage affecting the general fitness and survival of the organism or it is an asset for it. Plants exhibiting greatest degree of photoinhibition are evergreen perennials living in extreme environments (Osmond & Föster. 2008)

Furthermore, there is an objective difficulty in understanding the mechanism of photoinhibition and identification of the primary site of damage and the series of events. In other energy converting systems the primary driving force is of chemical nature and as that it can be quantified, added or removed according to the experiment's goal(s).

Moreover, the chemical substances acting as substrates, have specific binding sites within the system, which can be dissociated, analysed independently and finally reconstituted as a whole (Schagger, 2002). In contrast to other energy generating systems, in photosynthesis, the ultimate driving force is the energy of light, which unlike substrate molecules does not have one or at most a few 'binding sites', but instead it is absorbed by hundreds of chromophores, that all together cooperate to drive photosynthesis forward or to dissipate the excess of absorbed energy by different routes. The light can potentially affect all the components of the photosynthetic apparatus and their interactions, inducing thus secondary destructive reactions and repair processes.

7.2. Primary Sites of Photoinhibition.

The most common site of damage, resulting in photoinhibition, is PS-II D1 core sub-unit (Matto *et al.,* 1984; Barber & Anderson 1992; Aro *et al.* 1993; reviewed by Adir *et al.*, 2003; Edelman and Mattoo 2008). The phenomenon however, markedly at lower rates, can also result from inactivation of other PS-II proteins, viz., D2, cytochrome-b_{559}, CP43 & CP47 (Schuster et al., 1988; Yamamoto & Akasaka 1995; Zer & Ohad 1995; Ortega *et al.,* 1999). Along with PS-II proteins, hight light, especially when combined with low temperatures, has been shown to induce inactivation of PS-I reaction centres as well (Satoh, 1970; Inoue *et al.,* 1986; Sonoike & Terashima 1994). The resulted inhibition is due to a slow replacement of damaged PsaB core subunit (Sonoike 1996).

Furthermore, the reactive species damaging proteins in both photosystems, have also been reported (Nishiyama *et al.,* 2001), to affect a wide range of cellular components such as pigments, proteins, lipids, nucleic acids and even the constituent components of the repair cycle.

7.3. The mechanism of PS-II photodamage

In hight contrast to the primary site of photoinhibition, that as mentioned above is widely accepted to be the D1 protein, the mechanism inflicting *in vivo* damage, is still under debate (reviewed in Vass & Cser 2009). However, despite the lack of agreement on the nature of the chemicals that are causing damage and on the precise location of destruction, the general consensus is that the light-induced inactivation of proteins, is the result of excess 'excitation pressure', i.e., energy that has been neither utilised nor dissipated (Powels 1984; Kyle 1987; Demmig-Adams & Adams 1992; Asada 1994). It

has also been suggested that the damage to D1 protein is light-dosage effect, i.e. depends on the number of photons absorbed rather that the rate of absorption (Park *et al.,* 1995; Park *et al.*, 1996).

As agents causing photoinhibition through protein damage, are generally agreed to be the highly oxidising radicals, including Reactive Oxygen Species (ROS). They appear to be a natural concomitant of PS-II function. Generation and accumulation of reactive radicals ($P680^+$ & Y_Z^+) and ROS (e.g. hyperoxide - O_2^-; singlet oxygen- $^1O_2$; hydroxyl radical OH^-) is the result of disrupted electron transport (Barber & Anderson 1992; Aro *et al.*, 1993; Asada 1994), Thereby, photoinhibition, apart from light, that itself is a highly variable parameter, also depends on time of exposure, abiotic stress conditions and the metabolic demands of the organism at the moment (Huner *et al.,* 1998; Takahashi & Murata 2008). All these factors, as for instance CO_2 assimilation, growth, temperature, salinity, water availability, nutrient stress and others; individually or collectively, affect, in one or another way, the electron transfer reactions in thylakoid membranes and determine the magnitude of 'excitation pressure'. Consequently, photoinhibition as phenomenon is not confined to 'high irradiance', as it can occur, practically, under almost all light intensities.

Oxidative damage to the D1 protein can be induced by two distinct mechanisms operating under different experimental conditions: a) the acceptor (quinone) side mechanism which involves over-reduction of the plastoquinone acceptors and depends on the presence of oxygen, and b) the donor side mechanism, which is independent of the presence of oxygen and involves accumulation of the strong oxidants Y_Z^+ and/or P_{680}^+ (Jegerschold *et al.,* 1990; Theg *et al.,* 1986; Aro *et al.,* 1993; Barber, 1998). Lack of oxidised Q_A at the time of primary charge separation promotes charge recombination between reduced pheophytin ($Pheo^-$) and oxidized primary donor ($P680^+$), yielding an excited triplet state of P_{680} that in turn generates very toxic species of singlet oxygen $^1O_2$ (Macpherson *et al.,* 1993; Telfer *et al.,* 1994a; Telfer *et al.,* 1994b). Nevertheless, the evidence for $^1O_2$ involvement in D1 damage is not unambiguously conclusive (Sopory *et al.,* 1990; Flors *et al.,* 2006) and therefore, in these terms, its role in photoinhibition is still questionable.

In higher plants, acceptor side photoinhibition seems likely since increasing irradiance leads to progressive closure of PS-II reaction centres (Maxwell *et al.,* 1994). In contrast, acceptor side photoinhibition in cyanobacteria is somehow unlikely since most of PS-II remain open, even under high light illumination (Campbell *et al.,* 1995). Open PS-II reaction centres in cyanobacteria are thought to be due to high ratio of PS-I to PS-II (Fujita & Murakami 1987) and/or to shared electron pathways (PQ pool, cyt-b_6f complex) between photosynthesis and respiration (Shyam *et al.,* 1993).

Donor side photoinhibition is likely under conditions of insufficient / limited electron flow from the WOC to the oxidised primary donor leading to direct oxidation of the D1 protein from the long lived P_{680}^+ and/or Y_Z^+. While inactivation of the donor side does not usually occur *in vivo* under normal growth conditions, lowering the pH in the lumen due to fast electron flow under high light may lead to release of Ca^{2+} from the WOC resulting in down regulation of its activity (Krieger & Weis 1993). However, the exact molecular mechanism of this oxidative damage remains unclear until today as well as how under normal conditions PS-II minimises the possibility of P_{680}^+ oxidising the protein environment it is placed at.

ROS were theorised to cause photoinhibition via different modes of action, as for instance by altering the conformational state of the proteins and rendering them subject for proteolytic activity (Aro *et al.,* 1993), or by direct oxidation of amino acids adjacent to redox active cofactors in PS-II (Sharma *et al.,* 1997). The proposal for ROS being involved in D1 damage was made upon evidence showing antioxidants and scavengers of oxygen-free radicals not only inhibiting the light-induced damage of D1 protein, but in parallel to increase the photosynthetic activity in *Spirodela* plants (Sopory *et al*., 1990).

There has even been an alternative suggestion of indirect involvement of ROS in photoinhibition via suppression of D1 synthesis (Nishiyama *et al.,* 2004; Takahashi & Murata 2008), affecting thus the repair mechanism. To overcome the inhibition of D1 synthesis caused by ROS, plants, algae and cyanobacteria have been shown to respond to oxidative stress by increasing the abundance of natural antioxidants, e.g. tocopherol, in their cells under various light irradiance (Munne-Bosch & Alegre 2002; Foyer *et al*., 2006; Backasch *et al.,* 2005; Kreiger-Liszkay & Trebst 2006)

In spite however of the accumulated knowledge implicating the involvement of ROS in photoinhibition and their mechanism of action on D1 degradation and / or synthesis, the area remains an active field of research (Nishiyama *et al.,* 2004; Takahashi & Murata 2008).

Yet, regardless of the nature of the reactive species and their mechanism of action, the end-result of the light-induced damage to D1 is effectively a series of marked structural and functional changes at both, donor and acceptor sides in the RC of PS-II. (Baena-González *et al.*, 1999; Magnuson *et al.,* 1998; Haumann & Junge 1999)

7.4. Photoprotection & Repair Mechanism

The bewildering diversity of photosynthetic organisms, found almost in 'every nook and cranny' of this planet, implies at least one thing, astounding ability of photosynthetic apparatus for self regulation as a response to the ever changing environmental conditions. An ability that extends beyond the regulations adequate for efficient light harnessing to the faculty of protection from the detrimental effects of excess 'excitation pressure'

Along with a wide variety of features such as negative and positive phototaxis among motile photosynthetic bacteria and algae, reflective wax and light avoiding leaf and / or chloroplasts movements (Kasahara *et al.,* 2002) in plants, photosynthetic organisms have also evolved a series of fine, exquisite mechanisms at molecular and biochemical level to protect their photosynthetic machinery from the damaging effects of excess light. The best known and studied such mechanisms, quenching the excess of energy, are photorespiration (Osmond 1981; Kozaki & Tacked 1996; Park et al., 1996; Badger *et al.,* 2000); heat dissipation via xanthophyll cycle (Demmig-Adams & Adams 1992; Demmig-Adams & Adams 1996; Horton *et al.,* 1996;) and the consumption of reduction power via water to water cycle (Park *et al.,* 1996; Asada 1999)

To compensate for the inevitable loss from photoinhibition, photosynthetic organisms have evolved a repair mechanism that replaces the light-inactivated protein with a newly synthesized copy (Kyle *et al.,* 194; Ohad *et al.,* 1985; Schuster *et al.,* 1988; Arntz B & Trebst A 1986; Aro *et al.,* 1993). In respect that the repair cycle maintains in operation the required cellular level of functional reaction centers, the repair cycle can be regarded as part of the overall photoprotective mechanism. It is however significant

to emphasize that the repair mechanism itself is sensitive to abiotic stress conditions (Takahasi & Murata 2008), aggravating thus to certain extent the rehabilitation of photosynthetic activity.

Given the complexity of the reaction centers and the spatial organization of TM, this integral mechanism, is an intricate and astoundingly synchronized process, sensitized to any functional anomalies in photosynthetic apparatus. Based on research data, the repair cycle in plants, algae and cyanobacteria as well, is believed to include a series of well concerted sub-operations, of which the major ones are monomerization of PS-II dimers and partial disassembly of PS-II complexes, proteolytical degradation of the damaged sub-unit, incorporation of the newly synthesized copy into the sub-complex, reassembly of the whole complex of PS-II and finally reactivation of the electron transport through the repaired reaction centre (Kyle *et al.,* 1984; Vass *et al.,* 1992; Barber & Anderson 1992; Aro *et al.,* 1993; Melis 1996, Nixon *et al.,* 2005).

The Figure I.6., below is a hypothetical model for the stages that PS-II complexes undergo during the repair cycle.

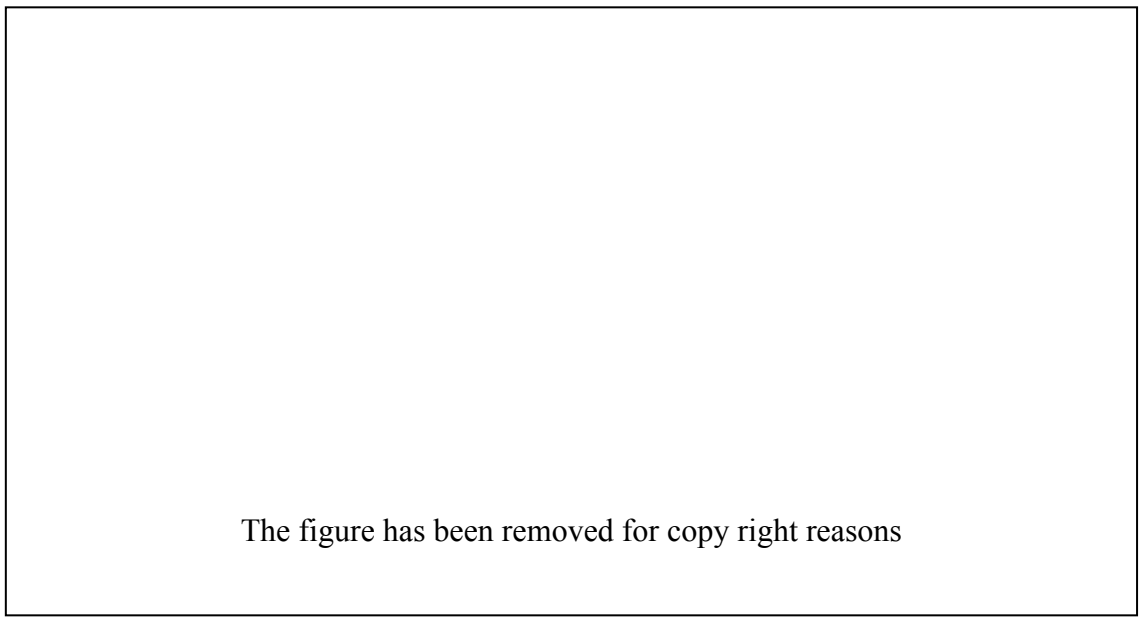


Figure I.6: **A hypothetic scheme for the operating repair cycle in cyanobacteria and in particular in *Synechocystis* 6803**. [drawn by J. Nield and P. Silva, Imperial College. Nixon *et al.,* 2005]. The repair cycle is a concerted multistep process. The key moments in this process, after the inactivation of the electron transport and damage of D1 subunit, are: (i) Monomerization of the dimeric PSII complex, (ii) Partial disassembly of PSII complex involving removal of CP43 subunit and the OEC, (iii) degradation of the damaged D1 subunit, and replacement with a newly synthesised copy (iv) re-association of extrinsic proteins and CP43, (v) carboxyl terminal processing of D1 subunit and rebinding of OEC followed by photoactivation of the complex.

In fact, the repair mechanism is even more complex, when considering other details of the process as for instance the lateral migration of PS-II components within the densely packed heterogenous TM during disassembly and preparations for repair, temporary storage of chromophores and cofactors of electron transport chain during disassembly, re-ligation of probably most of the pigments and cofactors, regulation of the extent of disassembly and many others aspects of this process that are still subject to research.

It has been suggested (Wu and Vermaas 1995; Funk & Vermaas 1999) that during the repair, pigments are being transiently bound to specific for the purpose Small CAB-like Proteins (SCPs) before being finally re-ligated to the RC subunits.

The fact that effectively D1 subunit is damaged most often than any other protein within the reaction centers, merits some more attention, especially through the lenses of photoprotection. The fact itself suggests that the damage is localized and thus the reactive species, concomitant of PS-II activity, are deactivated and therefore powerless to induce further damage to the reaction centre or elsewhere. On this basis, it is reasonable to suggest that this feature of photoinhibition resulting in D1 inactivation mainly, may be regarded as an evolutional advantage. Localized damage may ultimately be or just may be seen as part of photoprotective mechanism that prevents the diffusion of harmful reactive radicals to other than D1 proteins. Localized damage thereby is reducing not only the number of required enzymes for the repair, but above keeps the energetic cost for the repair at lower possible level and yet, does not aggravate the 'trafficking' within the heavily packed TM.

7.5. D1 core subunit - structure and posttranslational processing

PS-II core D1 subunit is characterised by phylogenetic conservation expressed at different levels: primary structure, rapid synthesis, N & C terminal posttranslational modifications and membrane orientation.

In all oxygen evolving photosynthetic organisms the D1 protein is synthesised as a precursor molecule (pD1), with a non-conserved lumenaly exposed carboxyl (C) terminal extension of 8-16 neutral and hydrophobic amino acid residues. In all cases the C-terminus of pD1 undergoes posttranslational modification by a lumenal peptidase, after which the last amino acid in the mature form of the protein, is alanine (Ala-344).

The functional significance of the D1 precursor and its C-terminus modification is still not fully elucidated. Mutagenesis studies have shown that this non-conserved extension is not a requirement for the integration of the D1 into the TM (Nixon *et al.,* 1992; Lers 1992). Studies on the LF-1 mutant of *Scenedesmus obliguus* which lacks the processing peptidase, showed that although the pD1 can be successfully integrated into the PS-II complex capable of oxidising Y_Z, it prevented however the ligation of the WOC (Nixon *et al.,* 1992). Nevertheless, despite the mutational studies, it is still not fully understood why this carboxyl terminus is retained throughout evolution in all photosynthetic organisms except for the *Euglena gracilis* (Karabin *et al.,* 1984) and some other species of dinoflagellates (Yamamoto *et al.,* 2001). The most reasonable explanation is that C-terminal modification controls the assembly of the D1 polypeptide into a functional PS-II complex.

In higher plants the N-terminal sequence in both core subunits D1 and D2 starts with formylmethionine which is removed, leaving threonine2 (Thr2) that is acetylated. These threonine residues can also be phosphorylated in both D1 and D2 although the importance of the process is not clear yet. In contrast to plant, the cyanobacterial D1 and D2 core proteins do not undergo N-terminal phosphorylation whereas reports for *Chlamydomonas reinhardtii* suggest that D2 but not D1 is phosphorylated.

The early folding models of D1 protein, based on hydropathy plots (Michel & Deisenhofer 1988) as well as the computer-generated models for the D1/D2 heterodimer using the PbRC as template, in line with the recent X-ray crystallographic studies of PS-II structure (Zouni *et al.,* 2001, Guskov *et al.,* 2009) conclusively show that both proteins span the TM with five transmembrane α-helices. In D1, the five transmembrane segments are designated with either roman numerals as I, II, III, IV, V or with block capitals letters A, B, C, D, and E. The protein is also characterised with two additional surface helices between III – IV (or C-D) and IV-V (or D -E) found on the lumenal and stromal sides respectively.

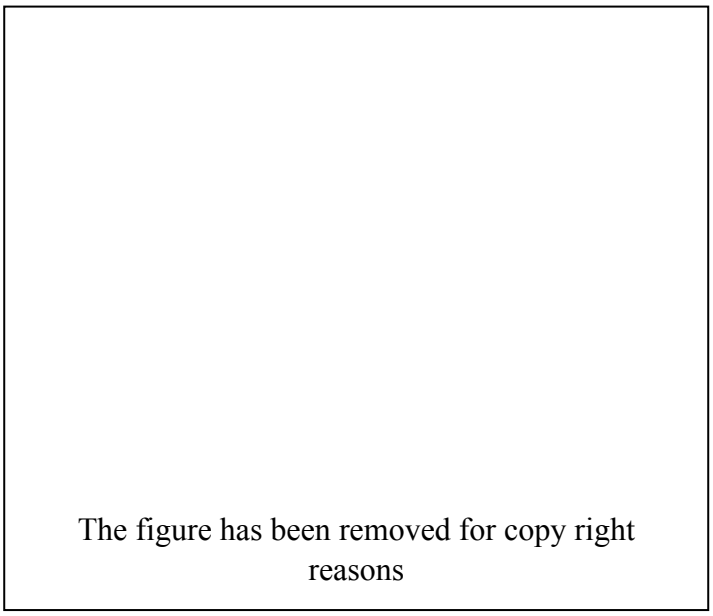


Figure I.7: **Schematic structural model of D1, showing the folding pattern of the protein.** Membrane-spanning helices are indicated in roman numerals I-V. There are also two more minor helices on either side of the TM. Also shown the putative positions of the bound cofactors and histidine residues. The picture has been reproduced from: http://macromol.sbcs.qmul.ac.uk/oldsite/psIIimages/D1.html

7.6. D1 turnover

The most striking feature of D1 protein is that it is being degraded and anew synthesised more rapidly than any other protein in the thylakoid membrane (Mattoo *et al.,* 1984; Ohad *et al.,* 1984). Despite the early discovery of the phenomenon the mechanism and reasons for this fast turnover were not clear. While the experimental data remain valid until today the interpretation for the fast turnover of this protein was not as valid. Of course one should consider that at the time of the discovery most of our knowledge about the structure and electron transfer reactions in PS-II were unknown. One of the early proposals (Kyle *et al.,* 1984; Ohad *et al.,* 1984) was that the degradation and *de novo* synthesis of this 32kDa protein, later to become known as D1, are responsible for the inhibition of photosynthetic activity. Now it is evident, that the fast turnover of the D1 protein is not the primary cause of photoinhibition, but merely a direct result of frequent photo-induced inactivation of the protein. In search for an interpretation for the rapid degradation of this protein, other than that of PS-II photoinhibition, an alternative proposal was formulated, the PEST hypothesis (Mattoo *et al.,* 1989). According to that, the degradation of the 32kDa protein comes as natural consequence of the presence of PEST (P-proline; E- glutamic acid; S-serine; T-threonine) like sequence, which is

common in unrelated proteins undergoing rapid degradation in non-photosynthetic tissues. However, experimental work did not provide support for the hypothesis. PEST like sequence, located close to the Q_B binding pocket is not required for D1 turnover (Tyystjarvi *et al.,* 1994; Nixon *et al.,* 1995).

The turnover of D1, as of any other protein is a very complicated process, since it consists of two fundamentally different, but nonetheless well synchronised and regulated types of reactions: degradation and synthesis. In reality the matters are even more perplexing when considering the complexity of the reaction centre, the impact of the overall electron transfer reactions, the heterogeneity of PS-II complexes, and the spatial organisation of the photosynthetic apparatus within TM. Furthermore, synthesis and insertion of *de novo* synthesised D1 protein must be well synchronised and adjusted to the rate of damage to PS-II RC, since the amount of D1 protein in the TM remains rather constant.

The rapid turnover of D1 is a natural concomitant of PS-II activity and occurs at all light intensities. The entire process is tightly controlled by light (Mattoo *et al.,* 1984), and is well preserved in all oxygen evolving photosynthetic organisms, from cyanobacteria to higher plants. However, comparing photoinhibition between cyanobacteria and eukaryotes, one should always bear in mind that unlike the metabolically specialised chloroplast functioning in a buffered microenvironment, cyanobacteria are fully autonomous cells, interacting directly with the surrounding environment. Yet, it is noteworthy that in cyanobacteria: the respiratory and the photosynthetic electron transport chain share the same PQ pool and the cyt-b_6f complex; accumulation of CO_2 is the result of an active mechanism to compensate for the lower affinity of their Rubisco for CO_2; there is no structural differentiation of TM as in chloroplasts into stacked grana, regions enriched in PS-II, and unstacked stromal membranes. It appears that cyanobacteria with their large PBS complexes rely on PS-II turnover, through the dynamic properties of the D1 protein, as an efficient means to restrict photoinhibition. In contrast, plants use antenna quenching in the LHC as an important means to protect the RC from the excessive light (Osmond & Föster 2008)

Key factor in the Repair cycle of PS-II is the rate of D1 turnover (Aro *et al.,* 1993; Anderson & Aro 1994). In higher plants the rate of D1 turnover is proportional to the

irradiance (Aro *et al.,* 1993) and reaches its maximum at saturating levels of photosynthesis. The limiting factor in D1 turnover appears to be the rate of degradation of this protein (Aro *et al.,* 1993). This limitation of PS-II turnover (due to degradation rate of D1), is ascribed (Anderson & Aro 1994) to the physical obstacle by granal stacking since it is likely that the repair of PS-II in plants does not occur *in situ* but in stromal lamellae (Aro *et al.,* 1993). In plants, synthesis of D1 protein occurs on ribosomes, bound to the stroma lamellae, implying the existence of translocating mechanism. However, the absence of 'free' mobile populations of D1 protein indicates that the newly synthesised copy of D1 does not migrate as a 'free' polypeptide but already incorporated into the PS-II RC. Early studies provided evidence that D1, D2, PsbO, and cyt-b_{559} were found in both TM and CM (cytoplasmic membrane) (Zak *et al.,* 2001), whereas the two core antennas proteins CP43 and CP47, found exclusively in TM indicating thus that the assembly of PS-II complexes is initiated in CM as PS-II RC-like, and completed in TM (Keren *et al.,* 2005).

In spite of the strong evidence indicating that the early stages of PS-II assembly take place in CM, there seems to be uncertainty about the location of PS-II repair, especially in cyanobacteria. Does the repair cycle operate *in situ*, namely in TM or it takes place in CM? The results of biochemical fractionation and immunoblotting studies are in disagreement. While the CtpA protease, (involved in D1 C-terminal maturation) appears only in CM (Zak *et al.,* 2001), the FtsH protease (proposed to be involved in degradation of photodamaged D1) is found only in TM (Komenda *et al.,* 2006). Along with FtsH, PC47 is also found in TM. PC47 is a sub-complex of PS-II containing CP47 but lacking CP43, and is unable to oxidise water. It is believed to be the disassembled PS-II required for the removal of damaged D1 (Komenda *et al.,* 2006). Furthermore, existence of a population of functionally different (impaired electron transfer to plastoquinone and limited antenna size) PS-II reaction centres, in stroma lamellae (Anderson & Melis 1983), which increases under photoinhibitory conditions (Neal & Melis 1991), is an indication that the stroma lamellae must be the repair zone of damaged PS-II reaction centres in higher plants.

7.7. Two forms of D1 protein

With minor exceptions such as pine trees (Lidholm *et al.,* 1991), plants possess one form of D1 subunit, encoded by a single *psb*A gene, in the chloroplast DNA. In contrast, cyanobacteria have two distinct forms of D1 subunit, referred to as D1:1 and D1:2 forms, which are encoded by small *psb*A multigene families.

The cyanobacterium *Synechococcus* 7942 contains three active copies of *psb*A gene, namely *psb*AI and *psb*AII/III encoding for D1:1 and D1:2 respectively (Campbell *et al.,* 1995; Golden *et al.,* 1986). There are also two *psb*D genes, but they apparently encode identical D2 proteins. Mutational studies reveal that while the two D1 forms differ only in 25 of the 360 amino acids, the D1:2 form alters significantly the intrinsic properties of PS-II reaction centres that appear to be more resistant to photoinhibition. PS-II reaction centres possessing the D1:2 form exhibit around 25% higher photochemical efficiency (Öquist *et al.,* 1995) than those containing the D1:1 form. The higher resistance to photoinhibition of PS-II with D1:2, compared to those with D1:1 is largely because of the higher photochemical quenching of D1:2 and/or faster recovery of PS-II (with D1:2) due to higher capacity of the repair cycle (Öquist *et al.,* 1995).

In principle, increased irradiance is the cause for D1:1/D1:2 interchange (Clarke *et al.,* 1993; 1995), but stress conditions as low temperature (Kulkarni & Golden 1994; Sippola *et al.,* 998) or light-sensing system responding specifically to blue light (Tsinoremas *et al.,* 1994) can also induce the *psb*AII/III expression. All these factors are not mutually exclusive. However excitation pressure on PS-II can also trigger the interchange between the two forms of D1, and appears to be the likely cause of the phenomenon in cyanobacteria (Öquist *et al.,* 1995). Lowering the temperature under constant irradiance results in increased excitation pressure on PS-II, since light harvesting and primary photochemistry remain unaffected, whereas the overall electron transport, enzymatic reactions, D1 turnover, are all slowed down.

A remarkable feature of the D1:1/D1:2 interchange is that the replacement of D1:1 with the D1:2 form triggered by increased excitation pressure is paradoxically transient. Maximum recovery of photosynthesis (measured as fluorescence and oxygen evolution) coincides with maximum D1:2 content, with a photosynthetic rate higher than that observed after shifting from low to high light conditions. Upon acclimation, the cells go

through the cycle of interchange between the two forms of D1 subunit, but this time replacing the D1:2 with D1:1 form (Öquist *et al.,* 1995). This is in line with the reactivation of *psb*AI expression under continuous high light (Kulkarni & Golden 1994). Despite the reactivation of *psb*AI and the ongoing interchange of D1:2 with D1:1, the expression of *psb*AII/III remains high, indicating the existence of post transcriptional regulation (Kulkarni *et al.,* 1992). After that, it is reasonable to say that the D1:1 form is not simply a low-light form, but rather light-acclimated form, whereas the transient D1:2 form is a pre-acclimation stress-inducible form.

7.7.1. Why the D1:1/D1:2 interchange in cyanobacteria

The general consensus is that the stressed induced interchange D1:1/D1:2, along with the increased synthesis of D2 (Bustos & Golden 1992) is part of a protective mechanism in cyanobacteria. The existence of small *psb*A multigene families in all examined to date cyanobacterial genera (Golden *et al.,* 1986; Bouyoub *et al.,* 1993) indicates the importance of the two forms of D1 subunit for these species, and suggests a conserved mechanism amongst these organisms. Unlike cyanobacteria, higher plants almost universally contain only a single copy of D1 subunit and the intrinsic susceptibility of their PS-II to excitation pressure (set by the redox state of the primary stable electron acceptor Q_A) is constant among plant species (Öquist *et al.,* 1992).

Comparison of cyanobacteria to higher plants with regard to the turnover of PS-II raises some important issues, as for instance: why chloroplasts retain only a single copy of the D1 subunit whereas cyanobacteria have two? Yet, why these two forms are only transiently interchanged when exposed to stress conditions? Even though cyanobacteria and oxygen evolving photosynthetic eukaryotes have evolved similar strategies to control the absorption, distribution and dissipation of excess energy, important clues on the aforementioned questions could be found in differences in TM aggregation and in organisation of the respective light harvesting complexes.

The plant PS-II, in contrast to cyanobacterial one, has been believed to be more protected from short-term excitation because of a heat-dissipating mechanism operating in their LHC-II antennas (Horton *et al.,* 2000; Horton & Ruban 2005). Such mechanism of non-photochemical quenching (NPQ) seems to be entirely absent from PBS, and all of the absorbed energy is transferred directly to the reaction centres. To counteract the problem, cyanobacteria are thought to react with changes in the populations of

functional and non-functional PS-II. The resulted photoinhibition due to excess of energy, triggers the exchange of D1:1 with the D1:2, and which in turn helps to counterbalance photoinhibition, both by a faster D1 turnover and by an increased intrinsic photochemical efficiency of PS-II. The discovery however of Orange-carotenoid protein (OCP) in *Synechocystis* 6803 (reviewed in Kirilovsky 2007) that is believed to dissipate thermally the excess of energy input but it is not universally distributed among cyanobacteria, although does not refute the soundness of the proposed hypothesis, certainly raises questions and accentuate the need for deeper understanding of the overall *modus operandi* of photoprotection.

In cyanobacteria, state transitions are an important mechanism in balancing the excitation energy between the two photosystems under different light conditions or in response to changing metabolic demands for ATP and NADPH (Miller *et al.,* 1991). Increased demands for ATP as during active accumulation of CO_2, induces shifts towards state 2 favouring thus excitation of PS-I (Miller *et al.,* 1991). Under normal conditions, the D1:1 form is the preferable one since it results in more extreme State 2.

7.8. Proteolysis of D1 subunit

Since its early stage, the proteolytic activity degrading rather specifically the D1 protein in light exposed thylakoid membranes, was ascribed to membrane bound protease(s) (Ohad *et al., 1985*; Reisman & Ohad 1986). The initial cleavage of the D1 subunit was demonstrated to take place at the stromal side of the thylakoids (Greenberg *et al.,* 1987) and the likely location of this site seemed to be a membrane parallel helix interconnecting the transmembrane IV (D) and V (E) α-helices.

Experiments performed with very high level of radioactively labelled D1 protein, demonstrated, that by the end of the photoinhibitory treatment the D1 subunit was completely digested, since no residue fragments or their adducts were detectable. Complete degradation of the fragments generated by the initial D1 cleavage, strongly indicates rapid proteolytic processing of the fragments involving several proteases.

Identification of the protease(s) involved in the degradation of photodamaged D1 subunits is still subject of active research. As yet, the identities of proteases along with chaperones involved in processes of degradation of damaged proteins and in stabilisation of PS-II complexes during the repair cycle *in vivo* are not fully elucidated

and remain subject of debate (for review see: Edelman & Mattoo 2008; Nixon *et al.,* 2010)

So far, large part of our understanding on the operation of the repair cycle in plants, relies mainly upon experimental data obtained *in vitro*. Based on such studies in isolated systems, it has been proposed (Spetea *et al.,* 1999) that D1 degradation is a two step multi-enzyme process involving a strategic primary protease responsible to recognise the inactivated complexes and for the initial cleavage of the damaged D1 subunit, and secondary protease(s) involved in the following cleaning up processes. The initial cleavage of the damaged D1 protein, leading to production of a 23-kDa N-terminal fragment and the corresponding ~10kDa carboxyl terminal fragment, takes place in the grana and it is a GTP-dependent process (Spetea *et al.,* 1999; 2000) whereas degradation of the two breakdown products of D1 subunit is an ATP and zinc dependent process. In this two-step D1 degradation process, the DegP2 protease, member of the DepP/HtrA family of serine proteases, catalyses the primary cleavage of damaged D1 in a stromally exposed region between helices IV and V, in a GTP-stimulated process (Hauβuhl *et al.*, 2001). After the initial cleavage, the two breakdown products of D1 protein are removed by some members of FtsH protease family (Spetea *et al.* 1999; Lindahl *et al.,* 1996; 2000). In this proposed model, the FtsH protease is involved in the removal of D1 fragments and not in the important primary cleavage event. However, the above-proposed mechanism for D1 degradation has only been assessed *in vitro*, and the true *in vivo* role of DegP2 remains unclear.

The potential involvement of DegP/HtrA proteases in D1 initial cleavage have been tested in cyanobacteria and in particular in *Synechocystis* 6803 which possesses three members of the above mentioned family, designated as HtrA (*slr*1204), HhoA (*sll*1679), and HhoB (*sll*1427). A triple *Synechocystis* 6803 mutant with all three genes insertionally inactivated, although sensitive to high light (Silva *et al.,* 2002) exhibited little effect on D1 turnover (reviewed by Nixon *et al.,* 2010).

The complete genome sequence of *Synechocystis* 6803 revealed the presence of four putative FtsH homologues, encoded by *slr*0228, *slr*1390, *slr*1640 and *sll*1463 ORFs. Two of them, namely those encoded by *slr*1390 and *slr*1604 appeared to be of immense importance for cell viability since insertional mutagenesis of these ORFs with drug resistance cassettes could not lead to complete segregation in clones carrying the

mutations (Mann *et al.,* 2000) The FtsH proteins encoded by *slr* 0228 ORF, although dispensable, exhibited and important role in the assembly of functional PS-I (Mann *et al.,* 2000) and more importantly in the early stages of the repair of photosystem-II (Silva *et al.,* 2003; Komenda *et al.,* 2006). Pulse-chase assays show that *slr*0228FtsH in particular, is essential for *in vivo* degradation of D1 protein. Also, studies with *Arabidopsis thaliana*, where FtsHases are represented as a nuclear multigene family of twelve copies (Adam *et al.,* 2001; Sokolenko *et al.,* 2002), showed that both *var*I (*fts*H5) and *var*2 (*fts*H2) mutants have impaired PS-II repair cycle (Bailey *et al.,* 2002; Sakamoto 2002; 2003; Kato *et al.,* 2009). In particular, *var*2 mutant exhibits similar pattern of D1 degradation observed in *slr*0228-FtsH less mutant in *Synechocystis* 6803 (Silva *et al.,* 2003; Komenda *et al.,* 2009). Consistent with the role of FtsH protease in the event of initial cleavage of damaged D1, is the fact that two FtsHases, *slr*0228 and *slr*1604, are found in His-tagged PS-II preparations of *Synechocystis* 6803 (Kashino *et al.*, 2002).

Increasing evidence, based mainly on studies in intact cells, suggests that the FtsH proteases are not restricted to the removal of the breakdown fragments but instead, they are essential players in the initial cleavage of damaged D1 subunits. The mechanism appears to be evolutionary conserved between plants and cyanobacteria. Summarising the accumulated knowledge over the repair cycle of PS-II in cyanobacteria and higher plants a few models for the repair mechanism have been postulated.

Alternatively to the proteolytic degradation of the damaged D1 subunit, it has been also proposed that the initial cleavage of D1 is due to light-generated ROS (Mishra *et al.,* 1994; Miyao *et al.,* 1995). This type of 'chemical degradation' has been proposed after studies conducted *in vitro* and under exposure to extreme conditions. Studies in both *Synechocystis* 6803 and *Arabidopsis thaliana* have shown that exposure of the respective FtsH mutants to extreme light, although resulted in inhibition of PS-II activity, did not lead to degradation of D1 proteins in contrast to the D1 of the wild type which was degraded under the same conditions. Nevertheless, non-enzymatic cleavage of D1 subunit cannot be excluded especially under more extreme conditions. It is more likely that the oxidations caused by the ROS do not normally induce a 'chemical degradation' but rather signal a specific proteolytic digestion regulated by the translation mechanisms (Komenda & Barber 1995).

A crucial moment in the overall repair mechanism is the location of the repair cycle, i.e., the repair zone. This question is still a matter of debate, especially for cyanobacteria, whereas in plants, increasing evidence suggests that the stromal lamellae is the repair site of Photosystem-II. Immunochemical studies in cyanobacteria demonstrated that the purified plasma membranes contain a number of proteins closely associated with the reaction centres of both photosystems. The D1, D2 and cyt-b_{559} core subunits have been found in plasma membrane (Smith & Howe, 1993; Zak *et al.,* 2001) suggesting that the cytoplasmic membrane may be the site of biogenesis of PS-II core, containing D1, D2, and cytochrome cyt-b_{559}.

8. FtsH proteases

In all living organisms, peptidases are essential for sustaining life under ever changing conditions. This is why, evolutionary distant organisms, despite the differences in their structures and metabolic life forms, all share a basic set of peptidases. The cyanobacterium *Synechocystis* 6803 contains nearly 60 peptidase encoding genes, comprising ~2% of its genome. In *Arabidopsis thaliana* close 300 genes are peptidase encoding, that makes up nearly 1.2% of its genetic potential (Sokolenko *et al.,* 2002). Many of these peptidases are ATP dependent. In *E. coli* for instance, there are several types of ATP dependent proteases such as Clp AP, ClpXP, HslUV, and FtsH. The latter is unique among the rest of ATP dependent proteases not only because it is essential for cell survival but also because it is the only one that is membrane-embedded (Tomoyasu *et al.,* 1992).

The FtsH proteases are membrane integrated ATP-dependent metaloproteases. They are members of the 'triple A' (AAA) family of proteins, which in turn consists a distinct group within the Walker-type superfamily of ATP/GTPases (Walker *et al.,* 1982; Kunau *et al.*, 1993). The most distinguishable feature of the AAA family of proteins is the existence of one or two copies of a conserved sequence of 220-250 amino acids, commonly known as the AAA cassette (Kunau *et al.,* 1993), located towards the carboxyl terminus of the protein, Figure I.8. The amino acid sequence of this cassette displays three major conserved domains, two of which are assigned to the ATP binding sites referred to as Walker A and Walker B motifs. The third highly conserved domain within the cassette is known as Second Region of Homology (SRH). The presence of the two Walker boxes is not common only for the AAA family but is widespread among

the members of the superfamily of ATP / GTP ases and this why their presence is regarded as necessary but not sufficient to identify a AAA member. The defining feature of the AAA family is therefore the second region of homology, which is located towards the C-terminus of the cassette (reviewed by Beyer, 1997).

The 'triple A' enzymes are involved in an extraordinary wide variety of cellular processes including cellular housekeeping, control of the cell cycle, protein degradation, regulation of gene expression and organelle biogenesis (reviewed by Beyer, 1997; Patel & Latterich, 1998). Nevertheless, their modes of involvement in these cellular processes are mostly unclear. Their localisation in the host cells are also diverse; some are integrated into the plasma membrane or into the respective membrane of chloroplasts and mitochondria whereas many others are soluble proteins (Confalonieri & Duget, 1995). Apparently they share no common biochemical feature. This broad biochemical diversity prompted Kunau *et al.,* 1993, to introduce the name AAA proteases standing for "**A**TPases **A**ssociated with diverse cellular **A**ctivities" reflecting thus the functional diversity of these proteins. This family is sometimes referred to as CAD (Conserved ATPase Domain) (Choi *et al.,* 1996; Sun *et al.,* 1996).

AAA proteins are widespread in all-living organisms (including Archaea), indicating that their functional divergence relies on the conversion of the chemical energy stored in ATPs into biological activity. The family of AAA proteases can be divided into subfamilies, with varying degrees of intra-relatedness, according to the extent of similarities in the amino acid sequence. Within each subfamily the level of conserved amino acid domains is much higher than all the other AAA representatives or even no similarity at all is observed between different subfamilies. These regions of similarities are actually the fingerprints of the subfamilies. However, there is no clue to the common function of AAA proteins, besides ATP binding and/or hydrolysis.

FtsHases are membrane embedded, ATP dependent, Zn activated proteases. They are ubiquitous in bacteria and Eukarya, but unlike other AAA protease have not been found in Archaea (Tomoyasu *et al.,* 1993; Swaffield & Puruggan 1997; Langer, 2000; Chapter VI). The high sequence conservation of FtsHases, in combination with their almost universal distribution among living organisms, strongly indicate, that these metaloproteases are essential for cellular functions; hypothesis that is substantiated by

the strong phenotypes associated with FtsH mutants in various organisms. For instance, deletion of *ftsH* gene in *E.coli* is lethal (Tomoyasu *et al.,* 1993; Tomoyasu *et al.,* 1995); in *Bacillus subtilis* causes severe growth defects (Deuerling *et al.,* 1997); in *Synechocystis* 6803 the FtsHases slr1390 and slr1604 appear to be of immense importance for cell viability (Mann *et al.,* 2000); whereas in humans any abnormalities in FtsH function lead to neurodegeneration.

Most available information on these proteases is based on studies on the *E. coli* FtsH and its three mitochondrial orthologues in yeast (reviewed by Langer 2000). In addition to the AAA cassette featuring in all members of the 'triple A' family of proteins, the FtsH proteases bear some functional, and structural distinct attributes. They are the only membrane bound enzymes capable of digesting both transmembrane and periplasmic segments of a substrate protein, yet they can combine chaperone like activities with proteolytic function.

The most characteristic features of their primary structure are: the two transmembrane helices located towards the N-terminus, while alongside the large carboxyl terminus is the AAA cassette and two other conserved domains. The transmembrane segments (exception the Yme 1p in yeast, spanning the membrane once) anchor the FtsH to the respective membrane, namely the plasma membrane in *E. coli* (Tomoyasu *et al.,* 1993), the thylakoid membrane in higher plants (Lindahl *et al.,* 1996), and the inner membrane of mitochondria in yeast. Spanning the membrane twice, FtsHases expose their both termini to the cytoplasm (Tomoyasu *et al.,* 1995; Shotland *et al.,* 2000) or to the stroma in chloroplasts (Lindahl *et al.,* 1996). The first of the two conserved domains mentioned above, is the catalytic site that contain the HEXXH binding motif. It is usually activated by the Zn^{2+} ion, although there is *in vivo* experimental evidence for functional association of *E. coli* FtsH with Fe^{2+}, Ni^{2+}, Mn^{2+}, and Co^{2+} ions (Herman *et al.,* 1995). After the catalytic site and close to the extreme end of the large C-terminus is the other conserved helical region, which is assumed to form the leucine-zipper-like coiled-coil structure (Figure I.8).

The terminology '**fts'** was introduced in latest seventies when Hitora and co-workers were trying to identify *E.coli* genes involved in cell division, by isolating conditional lethal mutants that formed long, non-specific filaments at the restrictive temperature.

These particular mutations that failed to septate and formed elongated filaments while cultivated at elevated temperatures, were designated as '*fts*': **f**ilament-forming **t**emperature **s**ensitive. The hypothesis was that cell division mutants of *E.coli* would grow as filaments due to the inability of the daughter cells to separate from one another. Studies revealed the presence of numerous such proteins required for proper bacterial cell division. At least nine such genes in *E. coli*, e.g. *fts*A, *fts*I, *fts*H, *fts*K, *fts*L, *fts*N, *fts*Q, *fts*W, have been shown to play an essential role in the process, and their products were found to localise to the division plane (Lutkenhaus & Addinall 1997). Most of these genes exact functions however are still not fully understood. The *fts*I gene was independently identified as an encoding PBP3 (Penicillin Binding Protein) in *E. coli* (Spratt 1975; 1977) involved in the synthesis and stability of cell wall peptidoglycan. The FtsZ protein that assembles into a ring at the future site of the septum of bacterial cell division is a prokaryotic homologue to the eukaryotic protein tubulin (Bi & Lutkenhaus 1991; Erickson 1998). The chloroplast FtsH peptidase was first identified immunologically in spinach thylakoid membranes and expression of its gene was demonstrated to be light dependent (Lindahl *et al.,* 1996)

For the history of FtsH it may be useful to add that the *fts*H gene was discovered independently through 3 different phenotypes. For this reason certain synonyms may be found in the literature (Narberhaus *et al.,* 2009). In particular: *tol*Z - for colicin tolerance; *hlf*B - because mutants show a **h**igh **f**requency of **l**ysogenization when infected with phage lambda, and apparently f*ts*H - for filamentous temperature sensitive.

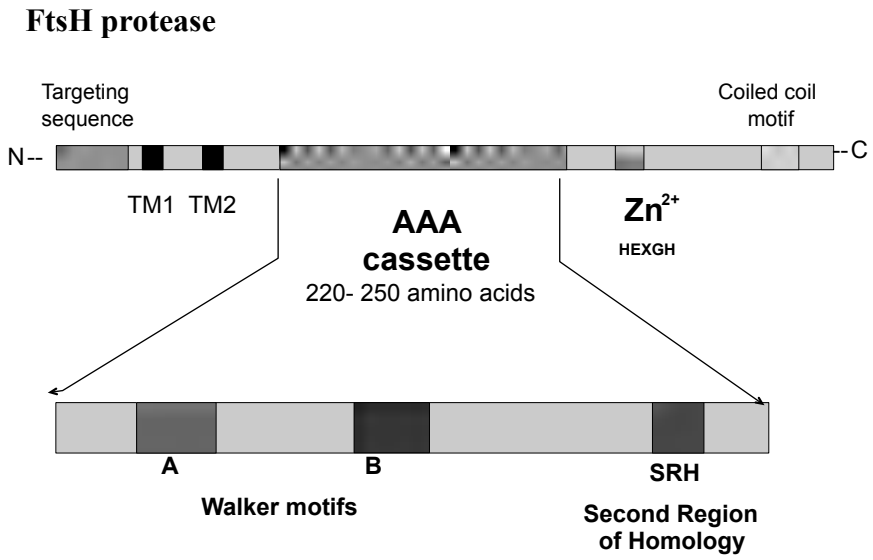


***Figure I.8*: Structure of FtsH proteases and the AAA cassette.**

FtsH proteases recognise as their substrate both soluble and membrane integral proteins that can be unfolded, misassembled or non-assembled polypeptides and therefore they constitute a quality-control system in the membrane (Akiyama 1994; Langer 2000). The quality control function of FtsHases requires specific recognition of non-native polypeptides, which suggests that these proteases have binding properties similar to those of molecular chaperones (Akiyama *et al.,* 1994). Indeed, FtsH enzymes can 'sense' the folding state of proteins interacting thus exclusively with non-native model proteins but not with their properly folded counterparts (Langer, 2000). Genetic evidence however suggests, that the chaperone like activities are independent of the proteolytic function of FtsH proteins (Arlt *et al.,* 1996; Rep *et al.,* 1996).

FtsH proteases, like other ATP dependent enzymes, exist in wild type cells of *E. coli* as multimeric complexes forming hexameric ring-like structures with the catalytic sites of their subunits buried within the central cavity (Shotland *et al.,* 1997; Krzywda *et al.,* 2002; Niwa *et al.,* 2002). Even though, every subunit appears to be in the same conformational state within the hexameric ring structure, the mode of ATP hydrolysis, either synchronised or sequential is not yet clear (Niwa *et al.,* 2002). Nevertheless, it is assumed that the active sites of FtsH subunits are self-compartmentalized, and thus excluded from the cytoplasmic environment. The amino-termini of FtsH subunits, in contrast to their carboxyl termini, seem to be essential for the FtsH-FtsH interaction during the process of homo-oligo-merisation (Akiyama *et al.,* 1995; 1999). The

mechanism that allows the FtsHases to digest both transmembrane and periplasmic regions of a substrate protein is not fully understood. It is not clear, how the hydrophobic, membrane-spanning domains of the substrate protein, are subjected to proteolysis, since the hydrolysis requires availability of water. Two working models for the destruction of membrane proteins by FtsH have been suggested; the shedding model and pulling model. The first requires the presence of two FtsH complexes from both sides of the membrane. The two FtsHases cleave off the exposed loops or domains of the substrate protein from either side of the membrane, thereby destabilising the hydrophobic part of the protein and making it accessible for further proteolytic degradation from the membrane surface (Leonhard *et al.,* 1996; Langer, 2000). In the pulling model, ATP hydrolysis promotes an active extraction of membrane embedded parts of the substrate protein and its translocation into a hydrophobic environment of the proteolytic site (Langer, 2000), followed by its degradation into small fragments.

The proteolytic mechanism mediated by FtsHases is elaborate and seems to be regulated by other factors. In *E. coli,* hetero-oligomeric complexes composed of FtsH proteins and two homologous polypeptides HflK & HflC have been identified on the periplasmic site of the plasma membrane. Mutational studies on *hfl*K and *hfl*C genes have demonstrated that these two proteins modulate the proteolytic activity of FtsH (Kihara *et al.,* 1996; 1997; 1998) and exhibit low but significant similarity to prohibitins, a highly conserved family of proteins in eukaryots.

The FtsH proteases in Bacteria play an essential role in cell homeostasis. In general, prokaryotic genomes appear to contain a single copy of *fts*H gene. Although numerous species contain two or more of these genes, in none of the bacterial phyla (excluding Cyanobacteria) multiplication of FtsHases seem to typify the entire phylum (Chapter VI). In cyanobacteria however, in high contrast to the rest of bacteria, multiplication of *fts*H genes, regardless of their precise number in each species, is a universal for the phylum phenomenon. Universality of FtsH multiplication characterises also higher plants, where the number of these proteases in each organism is increased even more (Chapter VI). The subcellular localisation and evolutionary relationship of eukaryotic with eubacterial FtsH proteases indicate that the eukaryotic FtsH-like proteins arose by gene migration events from an ancestral endosymbiont of chloroplasts and mitochondria to the primitive eukaryotic nucleus (Swaffield & Purugganan, 1997). Multiplication of

the FtsH proteins in cyanobacteria and higher plants correlates to certain degree with the evolution of oxygenic photosynthesis.

Most of the examined cyanobacterial species appear to have four FtsHases (more details in Chapter VI). This trend of multiple FtsH copies is manifested even stronger in higher plants. In *Arabidopsis thaliana* for instance 16 ftsH genes have been identified, number notably higher (Sokolenko *et al.,* 2002) than previously thought (Adam *et al.,* 2001). In higher plants, further multiplication of FtsHases has occurred after chloroplasts and mitochondria have diverged from their respective progenitors. Some of the *Arabidopsis thaliana fts*H genes, are more related to the yeast homologues rather than to cyanobacterial ones suggesting that those have derived from the progenitor of today's mitochondria. Eukaryotic orthologues of the FtsH-like proteins of *Synechocystis* 6803 have been identified using phylogenetic approach (Chen *et al.,* 2000). Three out four different FtsHases present in *Synechocystis* 6803 closely relate to the plant FtsH proteins, although whether or not the fourth FtsH of *Synechocystis* 6803 has a eukaryotic orthologue is not clear yet (Chen *et al.,* 2000; Chapter VI).

Surprisingly, four out of the sixteen *Arabidopsis thaliana* FtsH proteases are considered as proteolytically inactive (Sokolenko *et al.,* 2002), since they do not contain the zinc binding motif required for proteolytic activity (Leonhard *et al.,* 1996). These inactive subunits are referred to as FtsHi proteins. As yet, such inactive FtsHi forms have not been reported in cyanobacteria. The role of FtsHi proteins thus far remains a mystery. It has been proposed though, that they might be involved in chaperone like functions or could be part of FtsH homo/ hetero-multimeric complexes. Experimental evidence however has not corroborated the above propositions.

In eukaryotes FtsH orthologues are most often localised in mitochondria and chloroplasts (Langer *et al.,* 2000) and although encoded by nuclear genes, they are post-translationally transported to their respective organelles by specific N-terminal targeting sequences (Hugueney *at al.,* 1995; Leonard *et al.,* 1995; Chen *et al.,* 2000). Presumably some of the cyanobacterial FtsHases as well, might be involved in the respiratory chain. The multiplication of FtsH proteases in cyanobacteria and much more in plants can only be partially explained. Some of the FtsHases are targeted to thylakoid membranes in cyanobacteria or in chloroplasts in higher plants, whereas others reside in mitochondria.

The functional significance of having more than one FtsH protein involved in photosynthesis is not fully understood at the present. It has been proposed though, that the different *fts*H genes might be expressed under various environmental conditions as for instance with the *psb*A genes encoding for the D1 subunit. The hypothesis however, has not been experimentally substantiated. Obviously, there must be a reason that cyanobacteria and especially higher plants, maintain such a high number of different FtsH forms. Although homo/hetero oligomerization of FtsH polypeptides into multisubunit complexes (Akiyama *et al.,* 1995, Niwa *et al.,* 2002) is a prerequisite for their catalytic activity (Karata *et al.,* 1999), many aspects of the structural and functional importance of the FtsH subunits within the complexes are still ill defined.

8.1. Mechanism for D1 degradation catalyzed by FtsH

As mentioned earlier, FtsH proteases assembly into multimeric complexes, forming a hexameric ring-like structures, where the proteolytic site is located in the central cavity. This compartmentalization prevents aberrant degradation of cellular proteins since it creates a topological barrier between the non-target proteins and the catalytic site, which is accessible only to non-native polypeptides. Following recognition, the damaged D1 subunit is actively (ATP hydrolysis) translocated to the catalytic chamber of the FtsH-complex where it undergoes proteolytic degradation at the Zn centres. It has been proposed that this proteolytic reaction can be processed from either the N or C terminus (Chiba *et al.,* 2002) or alternatively from the ends generated after the initial cleavage event catalysed by FtsH. Initiation of the proteolysis from the amino terminus of the D1 subunit is very plausible, since it will allow synchronised removal of damaged D1 and co-translational assembly of a newly synthesised D1 copy (Zhang *et al.,* 1999). The studies of *Synechocystis* 6803 mutants, with D1 truncated at its N-terminus, are in favour of D1 proteolysis from the amino terminus (Komenda *et al.,* 2007). The N-terminus processing also correlates with the proposition that the phosphorylation of this terminus in plants controls the degradation of the subunit.

A characteristic feature of D1 degradation is the high specificity of the process since only the damaged D1 subunit is targeted for proteolytic degradation. This is consistent with the specific binding properties of FtsH proteases. The light-induced damages in D1 protein, unavoidably lead to conformational and functional changes in the subunit. All these alterations collectively, must leave this subunit in a relatively destabilised and

unfolded state, since FtsH proteases lack robust unfoldase activity (Herman *et al.,* 2003). The FtsH proteases recognise their target protein either because it has an accessible end specifying protease binding, or because the changes in the conformational state of the D1 subunit have resulted in exposure of an internal degradation signal. In this regard in particular, a highly conserved domain of 81 amino acids in FtsHases of oxygenic photosynthetic organisms (Bailey *et al.,* 2002) is worth more attention. This conserved segment might be involved in recognising damaged D1 and / or other PS-II subunits.

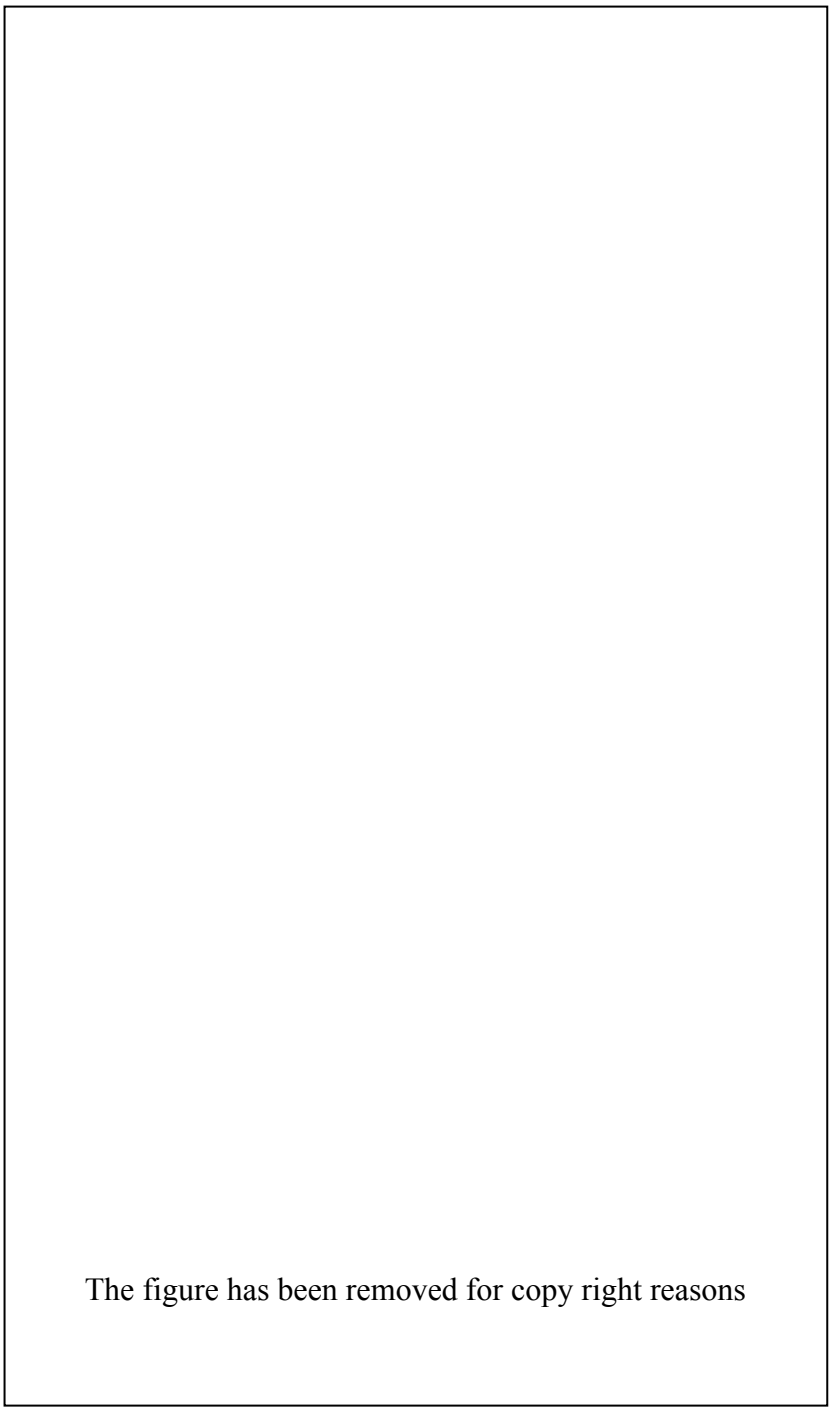


Figure I.9. **Hypothetical model for replacement of photo-inactivated D1 protein by the hetero-oligomeric FtsH complex.** The cartoon has been reproduced from Nixon *et al.,* 2010., and it is based on the postulated idea of FtsH being the sole protease involved in the degradation of D1 protein. This is a basic model were all external proteins and assembly factors have been omitted for clarity. A,B)Light-induced inactivation of D1 triggers the overall destabilization of the protein, that in turn initiates the repair cycle starting with monomerization of dimeric PS-II complexes, followed by partial disassembly to form RC47 complex. The FtsH complex recognizes its substrate protein from the amino-terminus of damaged D1 sub-unit and degrades the protein in 'a highly processive reaction' at its Zn activated catalytic domain. Pigments are suggested to be transiently attached to SCPs proteins until being re-ligated back into the new protein. C) Degradation of the damaged protein is well synchronized with the insertion of the new copy to minimize further destabilization of RC47. D) CP43 is re-attached to the complex allowing thus the reassembly of manganese cluster, followed by the final stage of dimerization of PS-II.

8.2. Investigating the role of FtsH proteases in photosynthesis

Photoinhibition, and in particular D1 turnover has been studied in great detail over the last few decades. Despite that, many aspects of the repair mechanism of this PS-II core subunit remain unclear, and many are still the subject of intense debate. It has not been unambiguously shown what are the primary causes of D1 inactivation. Where, especially in cyanobacteria, the repair of PS-II occurs, CM or TM? Is proteolysis of photoinactivated D1 protein a one or multienzyme process? These and many other aspects of the repair mechanism as for instance signalling continue to raise questions.

Yet, most of our understanding on this process comes from *in vitro* systems of eukaryotic chloroplasts derived from higher plants such as *Arabidopsis thaliana* and *Spirodela oligorrhiza* and green algae as *Chlamydomonas reinhardtii.* Substantial contribution to our knowledge on this subject, especially in intact cells, comes also from studies of the cyanobacterium *Synechocystis* 6803.

Thus, the cellular location of PS-II repair in chloroplasts, although widely accepted to take place in CM (based on biochemical fractionation and immunoblotting), has not been shown to operate *in vivo*. The same question for cyanobacteria remains thus far the subject of discourse. Some of the data from *in vitro* systems indicate cytoplasmic location (Zak *et al.,* 2001) for the repair cycle, while others, the thylakoid membranes (Komenda *et al.,* 2006).

Also, there seems to be lack of unequivocal acceptance for the number and nature of proteases involved in the process (for review see Edelman & Mattoo 2008; Nixon *et al.,* 2010). Thus, PS-II activity expressed as oxygen evolution, along with the degradation of D1 protein in FtsH2 less mutant in *Synechocystis* 6803, although notably impaired, none were totally blocked.

These, along with a series of other, well documented facts such as: omnipresence of FtsH proteases among living organisms (exception *Archaea*); high structural and functional conservation; indispensability or strong phenotypes in FtsH less mutants; multifunctionality; multigene family in oxygenic phototrophs; holoenzyme involving several FtsH proteases; raise some important questions.

Are the FtsH proteins the sole or the main proteases involved in the repair cycle of PS-II. If yes, then is this a universally patterned process in the repair of type-II RC. The latter question is of particular interest not only because of bewildering diversity of cyanobacteria alone, but also because *Synechocystis* 6803 where it has been tested so far has some unique features. Although derived from freshwater, it can also grow in sea water (Richardson *et al.,* 1983), and most importantly, this species can grow heterotrophically in the absence of functional photosystem-II (Pakrasi & Vermaas 1992; Anderston & McIntosh 1991).

Given the existence of several FtsH homologues and the *modus operandi* of the holoenzyme requiring at least two of them, there are questions that need to be answered. Which particular polypeptides will be included in the functional holoenzyme and what are the conserved features at the primary structure level characterising these proteases?

Bearing all these in mind, we have chosen to work with *Synechococcus* 7942, a cyanobacterial strain, that because of its morphological characteristics, and the relative ease of genetical manipulation, is well suited for *in vivo* observation with confocal microscopy of fluorescent tagged proteins or naturally fluorescent pigment-protein complexes. Use of FRAP technique (for details see Chapter II), can give data for real time dynamics of TM components, thereby providing important clues on the location of the repair mechanism in cyanobacteria.

Yet subjecting the *fts*H genes in *Synechococcus* 7942 to insertional mutagenesis and probing comprehensively mutants' phenotypes, can provide important evidence for a conserved role of FtsH proteases in the repair mechanism of type-II reaction centres. The taxon of *Synchococcus* is characterised by a large genetic diversity, and based on morphology (e.g. shape, swimming motility), physiology (e.g. pigment profile) and genetic traits (e.g. G+C % content) it is divided in Bergey's manual into five distinct clusters (Willamette & Herdman 2001). Along with *Prochlorococcus*, *Synechococcus* species contribute significantly to the global primary production and dominate the phototrophic picoplankton over vast areas of worlds oceans, with the latter being ubiquitous in all marine environments ranging from polar to tropical waters.(Scanlan & West 2002)

Furthermore, if deletion of FtsH protein from one strain (*Synechococcus* 7942) of this multi-species genus (*Syenchococcus*) will be shown to affect the repair mechanism, will then the lack of this protease affect the mobility of PS-II which has been shown (Sarcina *et al.,* 2006) that under certain circumstances can become mobile to facilitate its repair?

CHAPTER II

MATERIALS & METHODS

Chapter II

Materials & Methods

1. Bacterial species and growth conditions

1.1. Host strains for cloning. Growth and storage

In this project, *Escherichia coli* (hereafter *E. coli*) strains were used as host cells for cloning of DNA. All *E. coli* strains, listed in Table II.1, were grown at 37 ^{o}C in or on Luria Bertani (LB) medium (Sambrook & Russell 2001), supplemented with selective markers when necessary (for details see below). When grown on agar plates, LB broth (10gr Bacto-tryptone; 5gr Yeast extract and 10gr NaCl per litre) was supplemented with 1,5%(w/v) agar (LB Agar Miller, Fisher Scientific UK limited, Loughoborough, UK). Liquid cultures were grown at 37 ^{o}C in shaking incubator at 200rpm. The use of *rec*A^{-} host cells was a deliberate choice, since this prevents undesirable recombination between the insert and host chromosomal DNA and prevents multimerization of the plasmid population as well.

During transformation, *E. coli* cells were briefly incubated (30-60min) in rich medium, prepared immediately before use. This medium is known as SOC medium and it contains 2ml of filter sterilized 20% (w/v) glucose (or 1ml of filter sterilised 2M glucose) and autoclaved SOB medium to final volume of 100ml. Every litre of SOB medium contains 20.0gr of tryptone; 5.0gr of yeast extract; and 0.5gr of NaCl dissolved in deionised H_2O and supplemented with 10ml of filter-sterilized 1M $MgCl_2$ and 10ml of filter sterilized 1M $MgSO_4$ prior to use.

All preparations were carried from colony to colony. Liquid cultures were inoculated from a well-isolated colony on a freshly streaked plate. Methods for storing *E. coli* cells depended exclusively on the desired storage time. For short time preservation, i.e., one to three weeks, plates of streaked bacteria, sealed off with Parafilm® were stored upside-down at 4 ^{0}C. For long time storage, 20% (v/v) glycerol stocks in 2.0ml screw-cap sterilised vials, were stored at –70 ^{o}C.

Blue-white colony screening for recombinant plasmids is used when transforming the host cells (Table2.1) with a plasmid that provides α-complementation (pBluescripts vectors, pUC series vectors)

E. coli strains	Genotype background	Source / Reference
DH5α	*F- gyrA96(Nal^r) recA1 relA1 endA1 thi-1 hsdR17 glnV44 deoR Δ(lacZYA-argF) U169*	Woodcock *et al.,* 1989.
XL-1blue	*F' recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac*[F' proAB lacl^{q}ZΔm15 Tn10]. Genes on the F' episome are wild-type unless indicated otherwise	Stratagene / (Bullock *et al.,* 1987)
SCS 110	*rpsL(Str^r),thr leu endA thi-1 lacY galK galT ara tonA tsx dam dcm supE44(lac-proAB)*	Stratagene

***Table II.1. E .coli* strains used in this work.** Host genes descriptions: *ara* – mutation causes inability to use arabinose; *end*A$^-$ DNA specific endonuclease-I; *gal*K – inability to utilise glucose; *gyr*A – DNA gyrase subunit A; *lac*I – repressor of lac operon. LacI^q is a mutant that overproduce the repressor protein; *lac*Y – Lactose utilisation; *lac*Z – *β*-D-galactosidase; lactose utilisation. Cells with *lac*Z mutations produce white colonies in the presence of X-gal, wild type produces blue colonies; *pro*AB – mutants require proline for growth in minimal media; *rec*A – gene central to general recombination and DNA repair (mutations eliminates general recombination and renders bacteria sensitive to UV light); *sup*E – suppressor of amber (UAG) mutations. *thi*-1 – mutants require vitamin B1 (thiamine) for growth in minimal media; *dcm* – DNA cytosine methylase (mutations blocks methylation of cytosine residues); *dam* – DNA adenine methylase; *hsd*R – *E. coli* (or EcoK) restriction endonuclease (absence of this activity permits the introduction of DNA propagated from non *E. coli* sources). F$^-$ - strain does not contain an F episome; F' – strain contains an F episome.

Higher transformation frequencies of cyanobacterial species can be obtained when using plasmids that are protected from the host's restriction system by methylation (Elhai & Wolk, 1988). Therefore, cloning strains such as DH5α and XL-1 Blue that do not block methylation of adenine (*dam*) and cytosine (*dcm*) residues (Table II.1), were used for propagation of recombinant plasmids, consequently used for transformation of cyanobacterial strains. In contrast to DH5α and XL-1 blue, strains such as SCS110, which have both methylases (adenine, cytosine) inactivated, are particularly useful for construction of recombinants plasmids.

1.2. Cyanobacterial species

The majority of the cyanobacterial strains, used in the present project, have been obtained from the Pasteur Culture Collection (PCC) and the PCC numbering is used throughout the work.

All cyanobacterial strains used are listed in the Table II.2.

1.2.1. Growth conditions

Cyanobacteria were grown in BG11 medium (Castenholz, 1988) supplemented with 10mM $NaHCO_3$, 12mM sodium thiosulphate and 10mM TES buffer at pH8.2. When necessary, glucose at 5mM or antibiotics were added at the required concentrations (Table II.2).

Liquid cultures were grown at 30 ^oC in a shaking incubator at 100rpm, in 250ml flask as 100ml of culture in BG 11 medium, supplied with the appropriate antibiotic. An exception was the thermophylic cyanobacterium *Thermosynechococcus elongatus*, strain BP1, with the optimum growth temperature at 45 ^oC. All cyanobacterial strains used in this investigation were grown under constant illumination of white fluorescent light, with the light intensity adjusted to 10 μmol. $m^{-2}.s^{-1}$, unless otherwise stated.

For growth on solid media, BG11 was supplemented with 1.5%(w/v) agar (Difco, Maryland, USA) and all the strains were grown in a stationary incubator at 30 ^oC, apart from *Thermosynechococcus elongatus*, grown at 45 ^oC, under the same light conditions as for the liquid cultures mentioned above.

1.2.2. Preservation

For long time preservation, 200μl aliquots (in 2.0ml screw-cap tubes) of dense cell culture of cyanobacterial strains, mixed with the appropriate cryoprotective solution were plunged into liquid nitrogen and then stored at –70 ^oC. *Synechococcus* strains were mixed with 5% (v/v) DMSO (Dimethyl Sulfoxide), while for *Synechocystis* ones, 25% (v/v) glycerol were used instead.

Strain	Description	Growth	source
Synechococcus 7942 WT	wild type	BG11	PCC
Synechococcus 7942 ftsH^{--}	FtsH 260 less mutant	BG11 50µg/ml kanamycin	This thesis
Thermosynechococcus elongatus BP-1	thermophylic cyanobacterium, wild type	BG11	
Synechocystis 6803 WT (GT)	glucose tolerant wild type	BG11 with or without glucose	W. Vermaas, Arizona State University
Synechocystis 6803 ftsh0228 gfp	mutant with GFP tagged to FtsH0228	BG11 with or without glucose	Imperial College, London.
Synechocystis 6803 ftsh0228-less mutant	mutant lacking slr0228	BG11 50µg/ml kanamycin	University of Warwick.

***Table II.2*. Cyanobacterial species and strains used during this project.**

2. Molecular Biology Techniques

2.1. Reagents, buffers equipment.

All chemicals were analytical grade reagents purchased from Sigma Chemicals (St. Luis Missouri, USA) or BDH Chemicals (Poole, Dorset, USA). All buffers were prepared according to Sambrook & Russel (2001).

Plastic and glassware, as well as the most of the buffers used in molecular biology were autoclaved at 121 ^{o}C for at least 15 minutes. High temperature sensitive solutions were sterilised by filtering, using sterile, syringe filters with pore size of either 0.2µm or 0.45µm (NALGENE Labware).

Centrifugation of small volumes up to 2.0ml were performed in the "Biofuge pico" microcentrifuge (Heraeus Instruments, Germany) while for larger volumes up 50ml the benchtop centrifuge "Mistral 3000" (GMI Inc, Minnesota, USA) was used instead.

For pH regulation the "pH211 Microprocessor pH meter" (Hanna instruments Ltd, Leighton Buzzard, UK) was used

2.2. Mutagenesis strategies

This is just a brief outline of the strategy adopted in the present study, to inactivate the *fts*H genes in cyanobacteria. A large portion of the ORF (open reading frame) to be

investigated was amplified by PCR and ligated into the MCS (Multiple Cloning Site) of the cloning plasmid (pBlueskript SK or pBluescript KSII-). An antibiotic resistance gene, extracted from the respective plasmid, was inserted approximately in the middle of the PCR fragment, so at least 120bp of the ORF were left, flanking the drug cartridge on each side. The new recombinant plasmids, with the target genes disrupted by an antibiotic resistance cassette were used to transform the cyanobacterial species. Insertion of foreign DNA (recombinant plasmid) into the cyanobacterium to be investigated relied mainly on the natural competence of cyanobacteria, i.e. ability for uptake of exogenous DNA. However, alternative method such as electroporation has also been applied. Either way, once inside the cell, incorporation of the recombinant plasmid into the chromosomal DNA is achieved by an active double homologous recombination pathway that possibly involves the *rec*A gene product (Murphy *et al.,* 1990). When the antibiotic resistance cassette is flanked on both sides by sufficiently long (+80bp) sequences, derived from the chromosomal DNA, accurate homologous recombination can take place. However, the longer the flanking DNA, the higher the transformation efficiency (Williams *et al.,* 1988). Selection of potential mutants was based on resistance to the respective antibiotic.

2.3. Working with nucleic acids

2.3.1. Isolation of genomic DNA

Cyanobacterial genomic DNA was extracted from cells harvested from dense cultures by centrifugation. The cell pellet was resuspended in 400µl of sterile TES buffer (5mM Tris pH8.5; 50mM NaCl; 5mM Na-EDTA). Lysozyme (Sigma) was then added to a concentration of 10mg.ml^{-1} and the solution was incubated at 37 ^{o}C for 15 minute with occasional shaking to prevent cells from settling out. Samples were processed further, by applying the manufacturer's protocol for DNeasy Plant Mini Kit (Qiagen, Germany) from the addition of RNase A onwards. Purified DNA was eluted from the DNeasy Spin Column using 10mM Tris Buffer (pH8.5) and stored in 0.5ml tubes at –20 ^{o}C.

2.3.2. Isolation of plasmid DNA

Liquid cultures of *E. coli* strain containing the required plasmid were grown overnight at 37 ^{o}C usually in 10ml LB medium. The cells were harvested by centrifugation and the QIAprep Miniprep kit (Qiagen) was used to extract the plasmid DNA. Plasmid

DNA was eluted in 10mM Tris buffer (pH8.0) or in TE buffer (10mM Tris, 1mM EDTA, pH8.0) and stored at –20 ^{o}C.

2.3.3. Agarose Gel Electrophoresis and quantification of DNA

The technique of Gel Electrophoresis was used to visualise, and quantify DNA fragments. To prepare the gel matrix, agarose type II (Sigma, USA) was dissolved in TAE buffer (40mM Tris-acetate, 1mM EDTA pH 8.0) (Sambrook & Russel, 2001) by heating. The percentage of agarose used, dependent exclusively on the expected size of DNA to be resolved, ranging thus from 0.8 to 1.2 % w/v. Electrophoresis was carried out by applying electrical potential in the range of 50-100 volts. Visualisation of DNA fragments was carried out with pre-stained agarose gels (0.5μg.ml^{-1} ethidium bromide) under UV illumination in a UV gel documentation system (Chemigenius Bioimaging System, Syngene, Cambridge, U K) and printed by video graphic printer (Sony).

DNA sample preparation included addition of 6x Loading Dye Solution (MBI Fermentas) containing bromophenol blue (migrating with 300bp fragments) and xylene cyanol FF (migrating with the 4000bp fragments). The volume ratio of sample to Loading dye solution was 5:1, respectively.

For quantification of the DNA fragments as well as assessment of their sizes, the 1Kb DNA Ladder (New England Biolabs) was used.

2.3.4. Purification and precipitation of nucleic acids

Extraction of desired DNA fragments from an agarose gel, was performed by using the QIAquick Gel Extraction Kit (Qiagen, Germany), following the manufacturer's protocol.

DNA clean up applications, prerequisites for efficient downstream applications such as cloning, were applied to all enzymatic reactions as well as to confirmed PCR products by using the QIAquick PCR Purification Kit (Qiagen, Germany).

Concentration of diluted DNA solutions were performed by ethanol precipitation. For the purpose, the DNA solution was mixed with 3M Sodium Acetate (volume ratio 5:1 respectively) and 100% ice chilled ethanol (volume ration 1: 3 respectively). Left for at least 30 minutes on ice, the solution was then centrifuged at 4 ^{o}C. The pellet was resuspended in 70% ethanol and then centrifuged again at 4 ^{o}C to pellet. The DNA was

finally resuspended in an appropriate volume of either ultra pure water or TE buffer (10mM Tris, 1mM EDTA, pH8.0) to acquire the desired concentration.

2.4. Genetic engineering tools

2.4.1. Restriction enzymes

All restriction enzymes used in this work, are type II endonucleases and were purchased from New England Biolabs (Beverly, Massachusetts, USA). Single digestions were performed at optimal conditions (buffer, temperature) recommended by the manufacturer. The time of the reaction varied, the completion however of the digest was always confirmed by running a small sample on agarose gel. Double digest was also carried out when possible, i.e. availability of suitable common buffer and, same optimum temperature for the reaction.

2.4.2. Ligases, Polymerases and other modifying enzymes.

Formation of a phosphodiester bond between 5' phosphate and 3' hydroxyl termini in double DNA was catalysed by the ATP dependent T4 DNA ligase purchased from New England Biolabs. The ligase buffer subjected multiple freeze / thaw cycles may lose activity due to degradation of ATP, therefore T4 DNA ligase 10x buffer was stored at -20 ^{o}C in single use aliquots. There was considerable latitude in the temperature and time needed for a successful ligation. Efficient blunt end ligations were performed at temperatures around 16 ^{0}C and for 4-18 hours while sticky-end ligations were managed at room temperatures (~22 ^{o}C) for 3-6 hours. To confirm ligase activity a positive control of linearised vector with compatible cohesive or blunt ends and no insert DNA, was often performed in parallel with the main reaction. The molar ratio of linearised plasmid and DNA insert varied, but in most cases, either 1:1 or a 1:3 molar ratio of vector / insert worked well. DNA clean up applications (see above) on linearised plasmid and insert DNA as well as the estimate of their concentration by agarose gel electrophoresis were necessarily performed before setting the ligation. To convert the molar ratios to mass ratios for a linearised vector and an insert DNA of known sizes the following equation was used:

[ngr of vector x kb size of insert/ kb size of vector] x molar ratio of insert/vector = ngr of insert.

DNA modifying enzymes, such as Calf Intestinal alkaline Phosphatase (CIP) (New England Biolabs) were used prior to blunt-end ligations to prevent re-circularisation of the linearised vector. The reaction was carried out at 37 ^{o}C for one hour.

Gene amplifications by PCR were carried out by using the VentR DNA Polymerase, purchased from New England Biolabs. The particular enzyme is a high fidelity thermophylic DNA polymerase. The high fidelity DNA replication achieved by this enzyme derives in part from an integral 3'→ 5' proofreading exonuclease activity in VentR DNA Polymerase.

2.5. Gene manipulation and cloning.

2.5.1. Nucleic Acid Amplification

Particular cyanobacterial genes were amplified by Polymerisation Chain Reactions (PCR). For the purpose, a pair of primers, with similar melting temperatures (within 3-5 ^{o}C) and the length of the binding sites not exciding 24bp; was designed for each gene to be amplified. Presence of suitable restriction sites alongside the amplified ORF was also taken into account. All primers used in this study, listed in Table II.3, were produced by MWG Biotech (Ebersberg, Germany). PCR were carried out in "Robo Cycler – Gradient 96" (Stratagene) and "Techne Genius" model FGEN02TD (Techne Ltd, Cambridge, UK) thermocycles, using Vent DNA polymerase (for details see above).

Presence of PCR products was verified by agarose gel electrophoresis. Immediately after the electrophoresis the PCR products were purified using QIAquick PCR Purification Kit (Qiagen, Germany) and the agarose gel electrophoresis was repeated to assess the concentration of the PCR product. DNA samples were eluted in 10mM Tris buffer (pH8.0) and stored in 0.5ml tubes at –20 ^{o}C.

Primer / Gene	Sequence (5' to 3')
F-0734 / tll 0734	**ccatctcgggttcagaatttg**
R-0734 / tll 0734	**ggcagctgaatacgttgtctt**
F-260 /9360*fts*H	**cgagctcgagactctgccgatcctggtcatcg**
R-260 /9360*fts*H	**ctagaagcttgaggtcaagccttgctcttcatcc**
F-660 /403092660	**tcta-gagctc -gacaaagtgcaggtcaatctgc**
R-660 /403092660	**agta-ctcgag- gtctcttccgagaagtcacgc**
F-820 /403099820	**tcat-gagctc-gacagtctccct cgattactcg**
R-820 /403099820	**agta-ctcgag-cgatgtcagaagcggcacc**
F-3010 /403103010	**agat-ctcgag -ctacagcctattcatcgatcagg**
R - 3 0 1 0 / 403103010	**tcat -gagctc-gagcaatttcctcgagtagatcg**

Table II.3. **Primers used in this study**

2.5.2. Plasmid vectors

Several cloning vectors were used in this study as some of them provided the sequences of the selectable markers (antibiotics). All vectors were high copy number plasmids (origin of replication such as ColE1 and pMB1 for pBluescript vectors and pUC series respectively, allowed them to be under relaxed control), ensuring thus high DNA yield. All plasmids (cloning and recombinant) used are listed in Table II.4.

Plasmid	description/ construction	Reference / source
pBluescript SK +/-	cloning vector	MBI Fermentas
pBluescript KS II +/-	cloning vector	MBI Fermentas
pUC4K	plasmid carrying *km* cassette	
pUC18	cloning vector, used in control transformations	MBI Fermentas
pUC4Cm	plasmid carrying Chloromphenicol cassette	
pBSp1.1	plasmid carrying Spectinomycin cassette	
pBsEmV	plasmid carrying Erythromycin cassette	
pBSK- 0734	recombinant plasmid	This study
pBSK-0734Km^{S}	recombinant plasmid	This study
pBSK- 0734Km^{A}	recombinant plasmid	This study
pBSK-360	recombinant plasmid	This study
pBSK-360 Km	recombinant plasmid	This study
pBKSII-703	recombinant plasmid	This study
pBsKSII-703Cm	recombinant plasmid	This study
pBKSII-417	recombinant plasmid	This study
pBKSII-417Sp	recombinant plasmid	This study
pBKSII-745	recombinant plasmid	This study

Table II.4. **Plasmid vectors used in this work**. Abbreviations for antibiotic cassettes used in this table: km – kanamycin cassette, Cm - chloramphenicol; Sp - spectinomycin; Em - erythromycin.

2.5.3. Selection and screening for recombinant vectors

Genetic selection and screening methods used in this study relied on the expression (or non expression) of certain traits.

Use of chromogenic compounds, i.e. X-gal (5-bromo-4-chloro-3-indolyl-β-galactosidase) has been the basic method in screening for recombinant plasmids. When *lac*Z (β-galactosidase) expression was induced by IPTG (isopropyl-thiogalactosidase) in the presence of chromogenic substrate X-gal, colonies containing plasmids with inserts appeared white, while colonies containing intact plasmids appeared blue.

Selection (presence of sort of pressure during growth of host cells) relied on use of certain antibiotics; all listed in Table2.5.

Antibiotics	Solvent / Working concentration	Source
Ampicilin	Water / 50-100 µg/ml	Sigma-Aldrich
Kanamycin	Water / 50-100 µg/ml	Sigma-Aldrich
Chloromphenicol	Ethanol / 2.5 –25 µg/ml	Sigma-Aldrich
Erythromycin	Ethanol / 50 µg/ml	Sigma-Aldrich

Table II.5. **Antibiotics and their concentrations in respective growth media.**

2.5.4. Transformation of E. coli cloning strains

There are two methods to transform *E. coli* cells with plasmid DNA: electroporation and chemical transformation but the latter, which involves treatment of cells with divalent cations, has been exclusively used in this study. The produced competent cells are mixed with plasmid DNA on ice followed by brief heat shock and then short incubation in rich medium prior to plating on selective agar plates.

For chemical transformation, competent cells were either purchased (Stratagene) or produced in laboratory following the next protocol. Liquid cultures of *E. coli* strains were grown in SOB medium in Erlenmeyer flasks for 12-14 hours at 37 ^{o}C with moderate agitation. The cultures were collected into 50ml polypropylene centrifuge tubes and then chilled on ice for 10-15minutes. The cells were harvested by centrifugation for 12 minutes at 3000 r.p.m and at 4 ^{o}C. The cell pellet was resuspended by moderate vortexing in a volume of RF1 (100mM RbCl; 50mM $MnCl_2x4H_2O$; 30mM Potasium acetate; $CaCl_2$ x $2H_2O$ and 15% w/v Glycerol at final pH 5.8, sterilised by filtration) solution that was 1/3 of the volume collected followed by 15minutes incubation on ice. Cells were pelleted as before and resuspended in 4ml of RF2 solution (10mM MOPS; 10mM RbCl; 75mM $CaCl_2$ x H_2O; 15% w/v glycerol, final pH adjusted at 6.8, solution sterilised by filtration) followed by chilling on ice for 15 minutes. The produced competent cells were flashed frozen in liquid N_2 (aliquots of 1.0ml in 2.0ml screw-cap vials) and then stored at –70 ^{o}C.

Commercial competent cells (Stratagene) were transformed with ligation products following the manufacturer's protocol, while competent cells produced in the

laboratory, were used for transformations with plasmid DNA following the same protocol. A positive transformation control, using pUC18 plasmid was necessarily included to check for cell's competency. Often, mock transformations with no added DNA were also performed with small sample of competent cells inoculated onto solid LB agar plates with and without antibiotic to inspect antibiotic's activity and cells' viability respectively.

2.5.5. Transformation of cyanobacteria

Certain cyanobacterial strains such as *Synechococcus sp* 7942 and *Synechocystis sp* 6803 are transformable by exogenous DNA (Shestakov and Khyen, 1970; Grigorieva & Shestakov 1982). Although, the mechanism for natural DNA uptake is poorly understood, this characteristic feature of the above species has been proved to be a reliable means for their transformation and consisted the main way to accomplish this task in the present study.

For the purpose, cell cultures of *Synechococcus sp* 7942 were incubated at optimal growth conditions, i.e. at 30 ^{o}C in 50ml of BG11 medium in 150ml flasks, until the optical density (OD) at 750nm had reached 0.5. The culture was then diluted with 1 volume of fresh BG11 medium and grown overnight. Cells were harvested by centrifugation (at 3000 r.p.m. for 15 minutes) and resuspended in small volume of BG11 to adjust the concentration to 4 x 108 cells per ml. Aliquots of 100μl were transferred into 1.5ml tubes and mixed with the recombinant vector to be transformed. At this stage, cells with the DNA added were incubated stationary at 30 ^{o}C and under 10μmol.m^{-2}.s^{-1} of incident light for four hours as periodical gentle shaking of tubes was taking place. The cells were then transferred onto antibiotic free BG11 agar plates (~40ml) and allowed to recover for 2-3 days. Selection pressure, i.e. addition of antibiotic, was achieved by overlaying the agar plate with 2ml of cooled 0.6% agar containing sufficient amount of antibiotic to give the required final concentration. Colonies appeared 2-3 weeks after the addition of top agar with the antibiotic and were subsequently streaked onto antibiotic containing agar plates.

Gene transfer in cyanobacteria can be also accomplished by electroporation (Thiel & Poo, 1989; Muhlenhoff & Chauvat 1996), .i.e. reversible permeation of cell membranes by application of high potential voltage across the membrane. Electroporation along

with natural transformation method have been both applied to transform the thermophylic cyanobacterium *Thermosynechococcus elongatus* BP-1.

3. Biochemical techniques

3.1. Pigment content

For estimation of chlorophyll-α concentration by methanol extraction, cells in 1ml of culture were harvested by centrifugation at maximum speed 13,000 r.p.m for 5 min and resuspended thoroughly in equal volume of 100% methanol. The suspension was spun as before and the absorption spectra of the supernatant (methanol extract) were recorded (Porra *et al.,* 1989) in a spectophotometer (Hitachi U3310, Japan). The chlorophyll concentration was then calculated from the formulae:

Chlorophyll content in methanol: $\mathbf{[Chl]_{\mu g/ml} = (A_{665} - A_{750}) \times 12.56}$

$\mathbf{[Chl]_{\mu M} = (A_{665} - A_{750}) \times 14.0}$

As the equations in Porra *et al.,* 1989 are for organisms containing chlorophyll *a* & *b,* the above formulae are modifications of the originals (Porra *et al.,* 1989) befitting chlorophyll estimation in cyanobacteria that lack chlorophyll-*b.*

For estimation of pigments chlorophyll and phycocyanin in intact cells, room temperature absorption spectroscopy was used. For more details see in Biophysical Techniques.

3.2. Preparation of Samples for Transmission Electron Microscopy (TEM)

In order to investigate whether the deletion of *slr*0228-*fts*H gene in *Synechocystis* 6803, resulted in any abnormality in spatial distribution of thylakoid membranes, images of higher resolution, as those from Transmission Electron Microscopy were required. For the purpose, ultra thin specimens from the wild type *Synechocystis* 6803 and the mutant, grown under normal and high light conditions, were prepared.

Sample preparation for the microtome by embedding the cells in resin, is a several day protocol. 10ml of dense cultures were centrifuged and the pellet resuspended in 0.5% Gutaraldehyde in 0.1M Cacodylate. After 30 min, centrifuged again and the pellet was mixed with 3% Glutaraldehyde in 0.1M Sodium Cacodylate, and then left overnight at 4^{o}C for fixation.

To remove glutaraldehyde, samples were washed several times (centrifugation, removal of the supernatant and resuspension) with Sodium Cacodylate buffer. The samples were centrifuged again and the cells (pellet), were resuspended in 2% agarose solution kept at 40 ^{o}C. After gentle vortexing, cell suspensions were transferred into 1.5ml ependorf tubes and spun down for 3 minutes at 13000rpm in a microfuge. After discarding the supernatant, the pellet in the tube were left in ice for as time required (~1hour) for the agarose to set. The agarose, with the cells embedded in it was cut in smaller pieces and left for 3 hours to fixed with 1% Osmium Tetraoxide in 0.1M Sodium cacodylate.

The samples were centrifuged for 10-20sec, until lose pellet was formed. To remove the Osmium tetraoxide solution, the samples were washed with plenty of distilled water (for better results, left for 30 min in distilled water in a shaker). After washing, 0.5% Uranyl Acetate was added and the specimens were left overnight at 4^{o}C.

The next day, the Uranyl Acetate was removed and the specimens were dehydrated by following the next procedure:

- 30% Ethanol for 20 minutes at 4 ^{o}C.
- 50% Ethanol for 20 minutes at 4 ^{o}C.
- 70% Ethanol for 20 minutes at 4 ^{o}C.
- 95% Ethanol for 20 minutes at 4 ^{o}C.
- 100% Ethanol for 20 minutes at room temperature.
- 100% Ethanol for 20 minutes at room temperature.

Propylene oxide for 15 minutes at room temperature.
Propylene oxide for 15 minutes at room temperature.
Dehydration process was followed by resin infiltration and embedding with the next procedure.
Removal of propylene oxide and replacement with:

- Propylene oxide / resin in 2:1 ratio for 1 hour
- Propylene oxide / resin in 1:1 ratio for 1 hour
- Propylene oxide / resin in 1:2 ratio for 1 hour
- Resin for 1-2 hours on a rotator at low speed
- Resin for 1-2 hours on a rotator at low speed
- Resin overnight on a rotator at low speed.

The next day, the resin was removed, the specimens were transferred to dry capsules filled with fresh resin and left in an oven for 24 hours.

3.3. Measurement of PS-II concentration by ^{14}C-atrazine

This method relies on the ability of atrazine to bind specifically to Q_B site with molar ratio of 1:1. Therefore, measuring the amount of atrazine bound will provide an accurate measure of PS-II content (Chow *et al.,* 1990). Quantification requires use of radioactive ^{14}C-atrazine and is performed in a scintillation counter.

The cell cultures were concentrated to 25μM Chl by centrifugation and then resuspended in atrazine buffer (50mM TES; 10mM NaCl; 5mM $MgCl_2$ and 400mM sucrose, pH adjusted to 7.5). Chlorophyll concentration was measured by methanol extraction and the density of cell was estimated spectroscopically (A750).
Cell suspension was transferred in aliquots of 1ml each into eight screw-cap 2ml tubes. For control another eight screw-cap 2ml tubes with 1ml of atrazine buffer and no cells were also prepared. To estimate the background counts, a blank with 0.7ml atrazine buffer and 3ml scintillation fluid in a 5ml scintillation vial was also prepared.^{14}C-Atrazine stock (100μM in ethanol) was accurately diluted in atrazine buffer to 5mM. Then, 10, 20, 30, 40, 50, 60, 70, and 80 ml of atrazine solution were added to cell suspensions and controls as well and after mixing by vortexing, were left in the dark for 5 minutes. The mixtures were spun at maximum speed (13,000 r.p.m.) for 5 min. 0.7ml of each supernatant (samples and controls) with 3.0ml of scintillation fluid were added to scintillation vials. All, samples and controls were run for 5 min each in a scintillation counter (Perkin Elmer – 1414 Liquid scintillation counter).

Calculations of PS-II concentration is based on the principle that herbicide binding is reversible and thus evaluation of saturating level of atrazine will provide the concentration of PS-II. To create the saturation curve, we plotted the concentrations of atrazine added vs atrazine bound, while the final concentration of PS-II in the sample was estimated by linear regression from a double reciprocal plot, with correct weighting.

4. Biophysical Techniques

4.1. Spectroscopy

For consistency, all spectroscopic analyses were performed with cells grown under standard laboratory conditions unless otherwise stated. Namely for cyanobacterial species: 30 ^oC under white fluorescent light 10 μmol. $m^{-2}.s^{-1}$, in 250ml flasks as a 100ml

of culture in BG 11 medium, and supplied with the appropriate antibiotic at the required concentration

4.1.1. Absorption Spectroscopy

Room temperature absorption spectroscopy was performed using Hitachi U-3310 spectophotometer (Hitachi High-Technologies Corporation, Berkshire, UK).

To record the absorbance of cells suspensions, the spectrophotometer Hitachi 3001 was set as followed: 1) Method: Wavelength scan – Mode: Abs; 2) 750 -400 nm, 300nm/ min; 3) Baseline - User 1; 4) Delay: 0; 5) Slid width: 1nm; 6) Sampling interval: 1nm; 7) Peak finding: Integration method - rectangular; 8) Threshold: 0.01; 9) Sensitivity:1

Pigment concentration

For estimation of pigment content (chlorophyll and phycocyanin) in intact cells, absorption spectra at 750nm, 625nm and 678nm were recorded and the concentrations were calculated using formulae of Myers *et al.*, (1980). To correct for cell scattering, A_{750} was subtracted from A_{678} and from A_{625}.

Chlorophyll in whole cells (μM): **$[(1.0162 \times A_{678}) - (0.063 \times A_{625})] \times 1000/ 68$**
Phycocyanin in whole cells: (μM): **$[(1.0162 \times A_{625}) - (0.2612 \times A_{678})] \times 1000/ 111$**
Where 68 mM^{-1} cm^{-1} is the extinction coefficient of chlorophyll-*a,* and 111 mM^{-1} cm^{-1} is the extinction coefficient of phycocyanin.

4.1.2. Flash spectroscopy

Measurements of PS-I content in whole cell samples of *Synechococcus sp* 7942 were performed spectroscopically from the flash-induced oxidation and re-reduction of P_{700}. The device is custom-built by P. Rich (University College London). The method is designed to calculate the number of functional PS-I reaction centres.

As a light source, Xenon flash lamp (Perking Elmer Photometrics, Fremont, California USA) was used, and supplied with BG39 filters in the flash unit. To induce PS-I specific oxidation, and measure P_{700} activity, a wavelength of 703nm was applied. The latter wavelength specificity was achieved by using a 695nm cut-on filter and a 695-707 narrow-band interference filter on the photomultiplier tube.

To eliminate PS-II activity, prior to any measurements, all samples were supplied with 10μM DCMU [3-(3,4- dichlorophenyl) - 1,1- dimethylurea].

Successive oxidation and re-reduction states of P_{700} resulted from eight periodical flashes. The final result was an average of 10-20 transients.

Finally, the extinction coefficient of 64mM/cm (Hiyama & Ke 1972) was used to compute the concentration of P_{700}.

4.1.3. Fluorescence emission spectroscopy

Fluorescence emission spectra both at room temperature (RT) and 77K were performed using Perkin Elmer LS 55 Luminescence Spectrometer (Perkin Elmer, Maryland, USA).

For 77K emission spectroscopy the luminescence spectrometer was fitted with a sample holder containing liquid nitrogen. Samples with adjusted chlorophyll concentrations were injected into silica tubes with external diameter of 4mm.For room temperature emission spectroscopy, 3ml of samples, with adjusted chlorophyll concentration were added to fluorescence cuvettes. All experiments were repeated at least three times to ensure reproducibility.

The samples were regularly dark adapted for 5 min prior either to flash freezing in liquid N_2 or to RT emission spectra recordings.

All fluorescence emissions were result of excitation with a monochromatic light of 435nm or 600nm, exciting chlorophyll or phycocyanin respectively, and the fluorescence emission was recorded from 620 to 800 nm. Both excitation and emission slits were adjusted to 5 nm unless otherwise stated.

To produce graphs, the obtained data were edited with SigmaPlot 9.0 software (Jandel Scientific).

4.2. Fluorescence Recovery After Photobleaching - FRAP

The dynamics of photosynthetic membranes in cyanobacteria were investigated with the technique of Fluorescence Recovery After Photobleaching (FRAP) that used a laser-scanning confocal microscope (LSCM), Nikon PCM2000 (Nikon UK Ltd).

The basic idea behind the method (FRAP) is that a powerful focused laser beam that is kept stationary (not scanning) for a short time (few seconds) in a selected region of the cell bleaches that area. The intensity of the laser is then reduced and by switching again to scanning mode a series of images recording the development pattern of the bleached

spot can be produced and the diffusion coefficient (if any) can be calculated (Mullineaux *et al.,* 1997; Mullineaux 2004). The image is initially analysed using Nikon EZ 2000 viewer software, and then the one dimensional bleaching profiles are extracted with Image J (National Institute of Standards and Technology). To calculate the recovery rate (if any) and the diffusion gradient, the base-line fluorescence from pre-bleached cell is subtracted and Gaussian curve is fitted to the corrected profile using Sigma Plot 9 (Jandel Scientific). This gives the evaluation of the depth of the bleach and the diffusion coefficient is calculated by plotting bleach depth vs time using the following formula: $\mathbf{C_t = C_o R_o\ [R_o^2 + 8Dt]^{-1/2}}$ where C_t is the depth of bleach at time t; Co - the initial bleach depth; Ro - the initial half-width of the bleach, and Dt the diffusion coefficient (Mullineaux *et al.,* 1997*)*.

The main model organism of this course work, the cyanobacterium *Synechococcus sp* 7942, which has elongated cells with thylakoid membranes arranged in regular concentric cylinders running the length of the cell consists a good model system for FRAP analysis.

Samples were prepared by spotting a few microlitres (µl) of cell suspension onto BG11 agar plates, and allowed to dry so the cells were immobilised. The dry spots were then excised from the agar plate and sealed into a custom-made sample holder with a coverslip on top of it. The temperature was kept at 30 ^{o}C by circulating water from a thermostatically controlled water bath through a water jacket around the sample holder.

The excitation light was generated by different lasers producing monochromatic light of several distinct wavelengths, namely: 543nm from the 4mW Green He-Ne laser; 633nm from the Red He-Ne laser, and 457nm, 488nm, 514nm from the 100mW Argon laser

4.3. Oxygen electrode

During the course of this work, oxygen evolution measurements were performed using a Clark-type oxygen electrode, namely the Hansatech electrode (model DW2 for liquid phase or LD1 for gas phase; Kings Lynn, Norfolk, UK). The device was operated through a personal computer according to the manufacturer's instructions. Measurements were obtained from 1ml liquid cyanobacterial cultures grown under standard laboratory conditions unless otherwise stated. The cultures were gently

pelleted at 3800 rpm for 8 min, and resuspended in fresh BG11 medium to final chlorophyll concentration of ~0.008 mg/ml.

The oxygen electrode chamber was kept at constant temperature of 30 ^{o}C by circulating water from a thermostatically controlled water bath through a transparent water jacket which surrounds the chamber. The light was supplied either by a Hansatech internal light emitting diode or from an external light source reaching very high irradiance for the purposes of photoinhibition experiments. The intensity of incident light was either saturating or photoinhibing while to examine the influence of different wavelengths Lee filters (www.leefilters.com) were used. To ensure that O_2 evolution was not CO_2 limited, the liquid cyanobacterial cultures were supplied with 10mM of sodium bicarbonate.

As the results of oxygen evolution were initially calculated in μmol O_2/ml/min they were converted into μmolO_2 mg^{-1}Chl. h^{-1} and finally expressed as relative units to oxygen evolution at time zero. For consistency, all calculations and unit conversions were performed with Excel-spreadsheets (Microsoft) in specifically designed templates. The graphs were created using Sigma Plot 9 software (Jandel Scientific).

4.4. Measurement of Growth

Population growth was measured by following changes in the number of cells and was studied by constructing and analysing the growth curve.

4.4.1. Direct estimation of cell numbers

The number of cyanobacterial cells in population was measured by using a counting chamber of a hemocytometer. For the purpose, a 10:1 dilution was prepared and a drop of cell suspension was applied to the chamber slide while the optically flat coverslip was placed on top. Cell numbers were counted directly under a light microscope at 40x magnification. The average number of cyanobacterial cells in the squares was used to calculate the concentration of cell in the original sample. Since the space between the coverslip and slide is 0.02mm, and the grid contained 25 squares of a total area of 1mm2, the formula: cells per square x 25 x 50 x 104 gave an estimate of cells per ml.

4.4.2. Indirect measurements of cell number

For unicellular organisms the Optical Density (OD) at a specific wavelength, is proportional (within certain limits) to cell number. Therefore, turbidity readings can be

used as a more rapid method to estimate cell numbers. However as the light scattering properties are unique for every species, and yet, the light properties and settings in every spectrophotometer differ, we calculated the correction factor for *Synechococcus* 7942 and Hitachi3001 spectrophotometer. Based on Beer-Lambert Law and Using a hemocytometer, the correction factor was computed to be: $Fc=1.725 *10^8$

As a measure of cell scattering, the OD of 1ml of culture sample at 750nm wavelength was used, while the concentration of cells per volume of culture was calculated using the following formula: **[cells/ml]= A_{750}* Fc** (Where A_{750} is the absorbance value at 750nm wavelength and $Fc=1.725 *10^8$).

Yet, for consistency and to eliminate the possibility of human error all calculations of pigments in intact cells, throughout this course project, were made in Excel spreadsheets using the same templates.

CHAPTER III

CREATING FtsH-less CYANOBACTERIAL MUTANTS

Chapter III

Creating FtsH-less cyanobacterial mutants

1. Introduction.

The universal distribution of the membrane embedded FtsH metaloproteases in the domains of Bacteria and Eukarya, itself implies their significance for cell's homeostasis. Although their quantitative presence in both domains differs from taxon to taxon, in general they seem to be encoded by a single copy of *fts*H gene. Multiple copies of homologous genes in any taxa appear to be completely unrelated to each other (for more details see Chapter VI). Nonetheless, in Cyanobacteria and to larger extent in Green plants, these Zn-activated proteases seem to exist in multigene families and yet their multiplication is believed to have evolved in parallel with the evolution of photosynthesis to its oxygenic form.

The evolutionary mechanism of gene duplication allows organisms to acquire such redundant copies which are then free to evolve new functions, becoming thus homologous proteins involved in different processes. Some of these redundant copies in particular, appear to have evolved an important role in the quality control of thylakoid membranes of oxygenic phototrophs. Indeed, mutational studies in plants, *Arabidopsis thaliana* (Bailey *et al.,* 2002; Kato *et al.,* 2009) and in cyanobacterium *Synechocystis* 6803 (Mann *et al.,* 2000; Silva *et al.,* 2003; Komenda *et al.,* 2006) have provided evidence for implication of these proteins in housekeeping of TM. Thus, the *slr*0228-FtsH in particular, of *Synechocystis* 6803 have been shown to be involved in the assembly of functional PS-I (Mann *et al.,* 2000) and in the repair cycle of PS-II (Silva *et al.,* 2003; Komenda *et al.,* 2006) and in the removing damaged or misassembled PS-II subunits (Komenda *et al.,* 2006). The other two homologues, namely, *slr*1390 and *slr*1604, appeared to play even more crucial role as they were essential for cell survival, whereas the fourth copy, *sll*1463, exhibited no obvious phenotype under laboratory conditions (Mann *et al.,* 2000).

To probe the proposed theory of certain FtsH proteases being the key component of the quality control mechanism in thylakoid membranes, especially in that of the repair cycle of PS-II, we subjected the *fts*H genes in *Synechococcus* 7942 and *Thermosynechococcus elongatus* BP-1 to insertional mutagenesis. The genome sequences of the aforementioned cyanobacteria are available online. For more details about these two

species, genomes size and most importantly about the sequencing centres and the ID of each strain in some of the most widely used databases can be found in *Table III.1*.

As both species appeared to have four *fts*H ORFs in their chromosomal DNA, the first targeted genes were those that exhibited the highest similarity score with the *slr*0228 *fts*H in *Synechocystis* 6803. Selection of particular genes in *Synechococcus* 7942 and *Thermosynechococcus elongatus* BP-1 for insertional mutagenesis are explained in details in Chapter VI.

For the purpose of insertional mutagenesis a three step strategy was adopted. First, amplification of gene in question by PCR and cloning of this product into the Multiple Cloning Site (MCS) of a suitable plasmid vector. Second, disruption of the gene with a drug resistance cassette. Third, transformation of the cyanobacterium with the recombinant plasmid bearing the disrupted gene in question.

As cloning vectors, for the series of molecular biology manipulations, the pBluescript plasmids (pBlueskript SK- & pBluescriptIIKS-) were used. Along with the number of advantages they possess, e.g. blue-white colonies screening, most importantly they are suitable for the final stage of transformation of our chosen model organisms, as they are unable to replicate within the host cyanobacterial cells and consequently can not confer resistance to the applied antibiotic unless the targeted gene which is disrupted with the antibiotic resistance cassette will be incorporated via double cross-over recombination into the host's chromosome.

However, because cyanobacteria are polyploid organisms and under laboratory growth conditions may contain up to 7-10 copies of their circular chromosomal DNA, the isolated colonies of putative transformants were streaked out several times allowing thus complete segregation of cells carrying the mutated genomes.

Finally, to rule out the possibility of spontaneous mutations conferring resistance to the applied antibiotic and most importantly to confirm the full segregation, excluding thus the possibility of residual copies of unmutated genes left in some chromosomes a series of PCR analyses followed by restriction mapping were also performed.

	Synechococcus PCC 7942	*Thermosynechococcus elongatus* BP-1
Culture Collection / Synonyms	PCC 7942 / ATCC33912; *Anacystis nidulans* R2	BP-1
Sequence centre	Joint Genome Institute	Kazusa DNA Research Institute.
IMG Taxon object ID	637 000 308	637 000 320
NCBI Taxonomy ID	1140	197221
GOLD ID	Gs00319	Gc00096
Reference	Koksharova *et al.,* 2006	Field *et al.,* 2008
Genome Size	2,742, 269 bp	2,593,857 bp
ORFs	2719	2555
Protein Coding Genes	2662	2476
Isolation c.n,	By K Floyd 1973. California, USA	Beppu hot spring, Japan
Other info	Photoautotroph; Motile; Facultative aerobic; Mesophilic	Photoautotroph; Thermophilic

Table III.1. **Genome sequence centres and ID of *Synechococcus* 7942 and *T. elongatus* BP-1 in some widely used Databases.** IMG - Integrated Microbial Genomes; GOLD - Genome On Line Database; NCBI - National Centre for Biotechnology Information.

2. Targeting *fts*H genes in *T. elongatus* BP-1 for deletion.

The genome of *T. elongatus* BP-1 contains four *fts*H genes viz. *tll0131*, *tll0734, tll1832,* and *tlr0528* as annotated by the sequencing centre (Table III.1). *A*ll four of the ORFs are named as *fts*H genes and their products are annotated as cell division proteins. More details however about these genes, their encoded proteins and their IDs in some well known databases, for ease of reference or any other query, are in the following Table III. 2.

	Kazusa -Cyanobase **tll 0734**	Kazusa - Cyanobase **tll 0131**	Kazusa - Cyanoses **tlr0528**	Kazusa - Cyanobase **tll1832**
IMG gene ID	**637313349**	**637312735**	**637313142**	**637314462**
Product Name	Cell division protein - FtsH	Cell division protein - FtsH	Cell division protein - FtsH	Cell division protein - FtsH
DNA coordinates / size	755093..756988 (-) / 1896bp	110890..112728 (-) / 1839bp	527625..529484 (+) / 1860 bp	1913906..191580 (-) / 1935 bp
Amino acids sequence length	631 aa	612 aa	619 aa	644 aa
Transmembrane helices	Yes	Yes	Yes	Yes
Accession (NCBI)	NP_681523	NP_680922	NP_681318	NP_682622
External links ID	Gene ID:1011915 UniProtKB: Q8DKW7	Gene ID: 1010336 UniProtKB: Q8DMI5	GeneID: 1012188 UniProt: Q8DLG5	GeneID: 1010946 UniProt: Q8DHW1

Table III.2. **Four *ftsH* ORFs present in *T. elongatus* BP-1.** IMG home page: **http://img.jgi.doe.gov/cgi-bin/w/main.cgi**. Kazusa-cyanobase homepage: **http://genome.kazusa.or.jp/cyanobase/**

The highest similarity score to *slr*0228 *fts*H gene of *Synechocystis* sp. 6803 exhibited the *tll* 0734 ORF and therefore consisted the main target for insertional mutagenesis (for more details see Chapter VI).

2.1. Deleting *tll* 0734 *fts*H gene

The genomic DNA, extracted from dense culture of wild type cells grown in BG11 medium at 45^0 C, was used as a template for PCR amplification. The amplified part of the *tll*0734 ORF, 793 bp, was located approximately in the middle of this gene, Figure III.1.

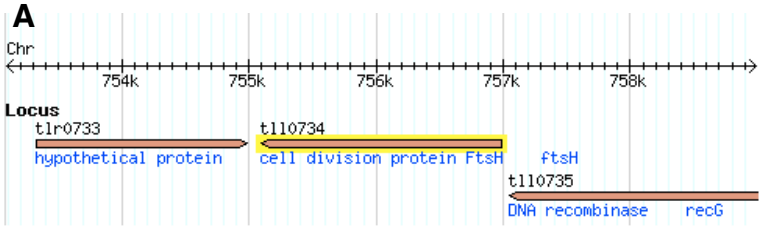
Confirmed by restriction mapping, the purified blunt-end PCR product was then inserted into blunt-end cutter EcoRV restriction site within the Multiple Cloning Site (MCS) of pBluescriptSK- cloning vector using T4 DNA-ligase. Prior to ligation however, in order to prevent re-circularisation, the linearised cloning vector was treated at 37 ^{o}C for an hour with Calf Intestinal Alkaline Phosphatase (CIAP).

Competent *E. coli* cells, strain DH5α, were transformed with the ligation mixture and planted onto LB agar plates containing the appropriate selective agents, namely

100μg.ml^{-1} of ampicillin, and the chromogenic compounds X-gal and IPTG to allow the expression of blue & white colonies due to α-complementation. The latter is achieved when the sequence of the MCS is disrupted, viz., when a foreign DNA fragment as the PCR product is inserted within the MCS.

In parallel with the main transformation, several controls were set as well. First, to confirm cells' viability, untransformed competent cells were streaked out on LB agar plates with no antibiotic added. Second, to exclude the possibility of a natural, or acquired resistance to ampicillin, or of any contamination of competent cells, mock transformation, no added DNA, was also performed on LB agar plates containing ampicillin at 50μg.ml $^{-1}$. Third, to confirm cell's competence, undigested circular cloning vector, pBluescript SK-, was used for transformation, resulting in blue coloured colonies only. Finally, to verify the absence of undigested or / and re-circularised vectors in the ligation mixture, competent *E. coli* cells were transformed with linearised plasmids.

A



B

ATGAAAGTCTCTTGGAAAACTGTTCTCCTTTGGTCAATCCCCCTACTGCTCATTGGGATTCTCCTGTGGC
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CAGAAATGGATGGCTTTGAGGGGAACACAGGCATCATTGTTATTGCTGCAACAAACCGTCCCGATGTTCT
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CGTTTGGCAATTCTCAAGGTGCACGCCCGCAACAAAAAACTGGCGCCCGAAGTGTCCTTAGAAGCCATTG
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TGGTGAAGAGTTCCGTCAGATTGTTGCTGAGTACACCGTAGTTCCCGATAAAGAGCGATTTGTGCCCCAA
CTG**TAG**

Figure III.1. **Design for the disruption of *tll*0734-*fts*H ORF in T. elongatus BP-1**. A) *tll* 0734 ORF, located on the complement strand of chromosomal DNA. B) DNA sequence of *tll* 0734 ORF. Start & Stop codons of the ORF are in bold black. In blue-amplified by PCR fragment, including the binding sites of primers, red highlighted text, flanking the blue letters . The highlighted, red letter text in the middle of PCR fragment is the restriction site of MscI for cloning of Km^R cassette.

For purity of potential transformants appearing as white colonies, a second round of selection was performed. For the purpose, white colonies were carefully picked off the plates and streaked out again onto LB agar plates with the same selective markers and chromogenic compounds (ampicillin, X-gal). Liquid cultures were then set for preparation of both, plasmid DNA and frozen stock for any future applications.

The generated new recombinant plasmid, designated as pBSK-0734, Figure III.2., was subjected to restriction mapping to examine the size (expected ~3.75Kb), and because of blunt-end ligation to establish the orientation of the PCR product within the MCS.

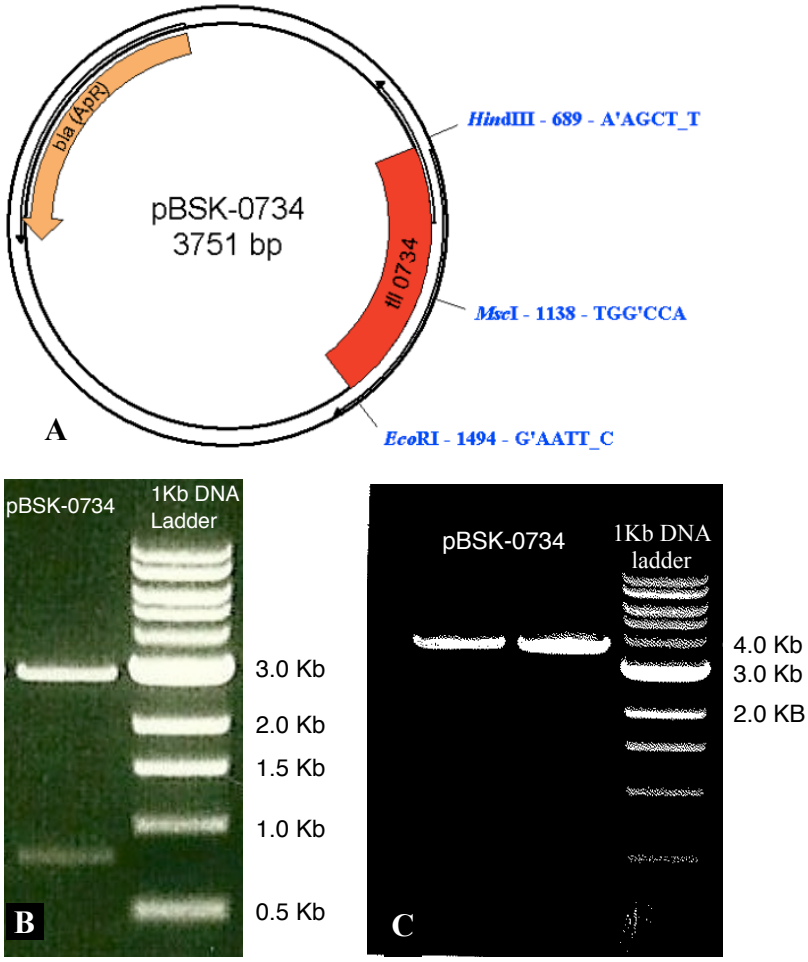


Figure III.2. **The recombinant plasmid pBSK-0734, and restriction map.** A) A drawing of the recombinant vector pBSK-0734 showing the *bla* (ApR) ORF coding for *β*-lactamase, that confers resistance to ampicillin; Part of *tll* 0734 ORF amplified by PCR, 793 bp and the restriction sites of endonucleases EcoRI, HindIII, and MscI. The drawing was created with scientific software pDraw32 designed for molecular biology applications. B&C) Images from Agarose Gel Electrophoreses of pBSK-0734 digested with different endonucleases: B) with EcoRI & HindIII combined yielding two bands. 805 and 2946 bp and C) with MscI that linearises the plasmid.

For interruption of the *tll*0734 fragment, in pBSK-0734 plasmid, the Kanamycin resistance cassette (Km^R) was used. The plasmid pUC4K, which bears the drug cartridge in question, was digested with HincII endonuclease yielding thus three bands as observed by agarose gel electrophoresis, Figure III.3. The middle fragment of approximately 1.2 Kb in size, which consists the kanamycin resistance gene, was

excised from the agarose gel and subsequently quantified by a second run of agarose gel electrophoresis for the forthcoming application of ligation.

The new recombinant plasmid pBSK-0734 was cleaved approximately in the middle of the *tll*0734 PCR fragment with the MscI enzyme, Figure III.2., generating blunt ends. Prior to quantification by agarose gel electrophoresis, the linearised pBSK-0734 was again treated with CIAP (calf intestinal alkaline phosphatase) to prevent the blunt-ends from re-ligating.

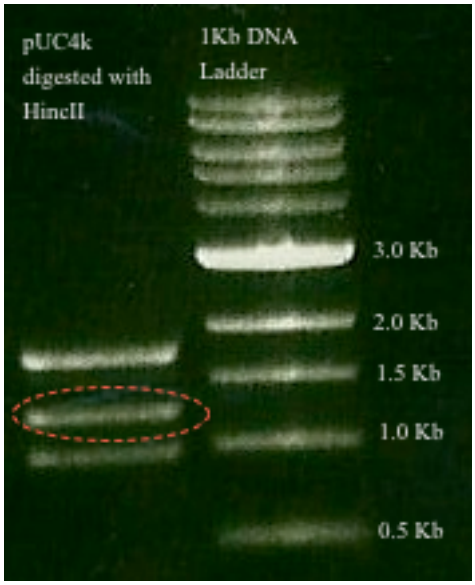


Figure III.3. **Agarose Gel Electrophoresis of pUC4K plasmid digested with HincII endonuclease**. The middle band of ~1.2 Kb is the desired gene conferring resistance to Kanamycin.

The final ligation of the blunt-ended kanamycin resistance cassette (Km^R) and the linearised pBSK-0734 was performed with molar ratio of insert to plasmid DNA at 3:1 for 16 hours and at 15 ^{0}C.

Ultimately, competent DH5α *E. coli* cells were transformed with the ligation mixture along with the control sets described previously. The selection of the desired transformants this time, was based on double antibiotic, namely Ampicillin and Kanamycin at concentrations 100μg.ml^{-1} and 50μg.ml^{-1} respectively. Several well shaped colonies were picked to inoculate liquid cultures for plasmid extraction.

To confirm the appropriate size of the final construct, expected at ~5.0 Kb, Figure III.4., and to define the orientation of the Km^R within the *tll*0734 PCR fragment, possibility due to blunt-end ligation, a set of digestions with HincII, StyI and XhoI endonucleases was performed Figure III..4. The restriction mapping revealed that two new recombinant plasmids were produced, both with the same size and orientation of *tll* 0734 fragment (Figure III.4 A &B), but with different orientation of kanamycin ORF within the disrupted gene, Figure III.4 C,D & E. The plasmid with the complimentary orientation of Km^R was named as antisense, hence pBSK-0734KmA, whereas the other with sense orientation as pBSK-0734KmS.

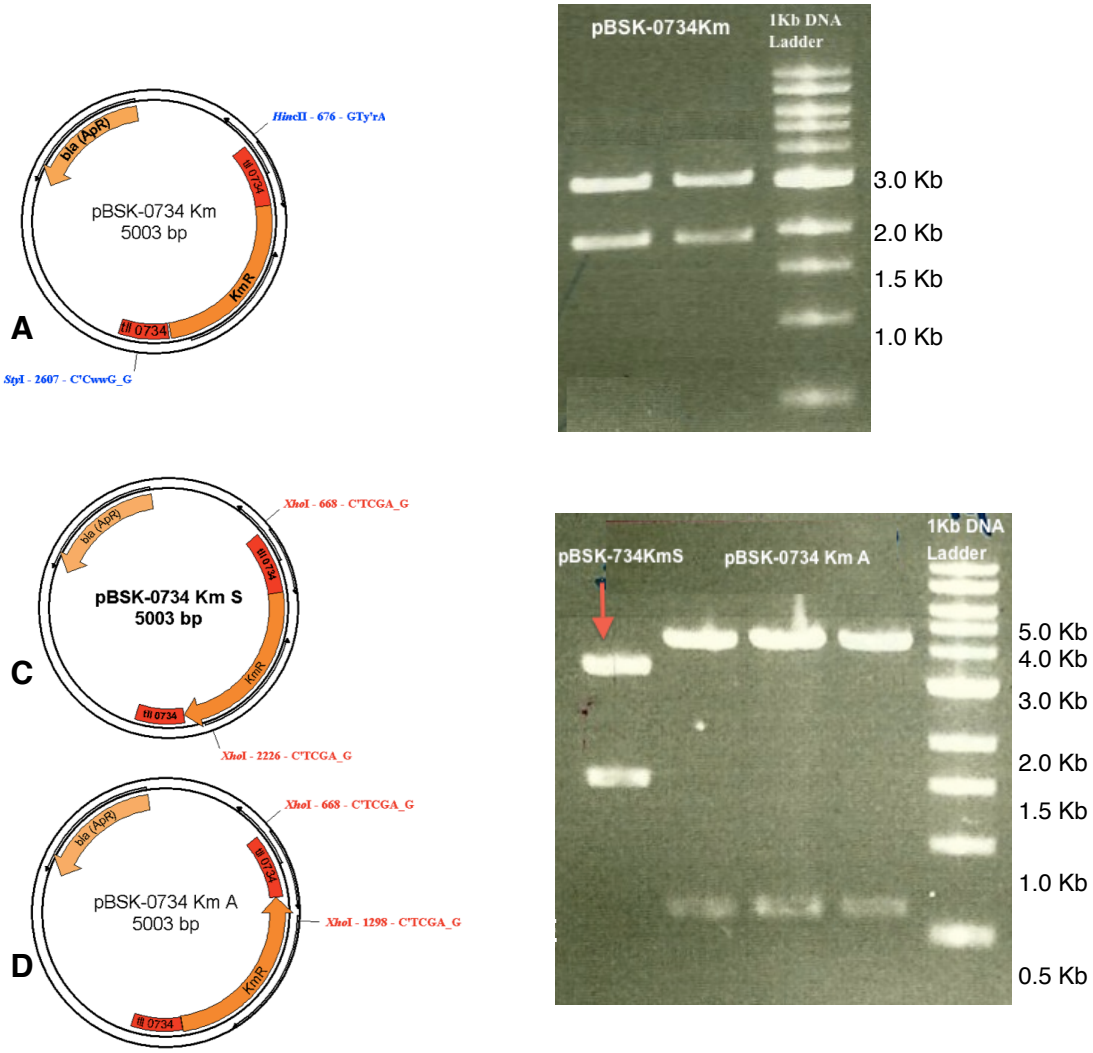


Figure III.4. **Restriction mapping of pBSK-0734 Km.** After successful disruption of *tll* 0734 with Km^R cassette, plasmids from several colonies were isolated and analysed. A & B) Cleaving the new recombinant plasmid with mixed HincII + StyI endonucleases confirmed once again the sense orientation of *tll*0734 PCR fragment within the cloning vector. Hinc II and Sty I cleave the plasmid at 676 and 2607 sites respectively, generating thus two bands, 1931 bp and 3072 bp. C, D & E) To define the orientation of Km^R ORF the endonuclease XhoI was selected which cleaves the plasmid twice, one within the MCS and the second within Km^R**.** Therefore the generated two bands will have distinct lengths for each orientation. Thus for sense orientation 1558bp and 3445bp whereas for antisense orientation: 4373bp and 630bp.

2.2. Transforming *T. elongatus* BP-1

Despite the consistent and prolonged efforts using two main types of transformation methods, natural DNA uptake, and electroporation, with numerous little variations, and making use of both constructs (pBsK-0734KmS, pBSK-0734KmA) at wide range of concentrations, the attempts of generating *tll*0734FtsH-less mutant in *T. elongatus* BP-1 met with no success.

Transformation, based on the ability of *T. elongatus* BP-1 for natural DNA uptake reported by Onai *et al*, 2003, resulted in some curious phenomena. Dense cell suspensions were mixed with various amounts of recombinant plasmid dissolved in TE buffer (Tris-HCl buffer at pH8.0 and EDTA) and incubated at 45^0C for 4 hours under constant dim light which prevents cell division. Subsequently, the mixtures of cells with recombinant plasmid were spread on BG11 agar dishes and incubated for 24-48 hours at 45^0C. After the recovery, antibiotic pressure (kanamycin) was applied, either overlaying the cells with 0.6% w/v agar (containing the antibiotic) or pipetting the aqueous solution of kanamycin under the agar medium. In several occasions, the appeared small green colonies proved to be impossible for further propagation. Streaking onto new agar plates with different concentrations of kanamycin always resulted in cell death. Inoculating liquid cultures however, seemingly promising in the beginning, was always ending up in formation of cell clumps and ultimately death.

Although *T. elongatus* BP-1 is a thermophilic cyanobacterium and thus suitable for X-ray crystallography studies, wide use of the species for genetic mutagenesis studies has been hampered to a large extent by the absence of a standardised transformation protocol. Fact that is easily discernible when considering that the largest part of published literature about the 3D structure of PS-II has derived from *T. elongatus* BP-1 (Zouni *et al.,* 2001; Guskov *et al.,* 2009*)* whereas the mutational studies of photosynthetic apparatus, its performance and regulations are in fact, derived from other model cyanobacteria species, amongst which, one of the most popular is *Synechocystis* 6803.

3. Targeting the *fts*H ORFs in *Synechococcus* 7942.

The DNA sequences of *Synechococcus* 7942 (for details Table III.1.) revealed the existence of four ORFs, all encoding FtsH putative proteins (for more details Table III. 3.). The four particular *fts*H genes, corresponding to the following Gene object IDs of the Sequencing Centre, namely: 637799745; 637799417; 637799360; 637798703; henceforth will be referred, for brevity reasons solely, with their last three digits. In particular: 745-FtsH, 417-FtsH, 360-FtsH and 703-FtsH. Details about their orthologous proteins in other photosynthetic species are in Chapter VI.

For the purpose of this study, all four of the aforementioned *fts*H genes were subjected to insertional mutagenesis and the same three step strategy as in case of *tll*0734 of *T. elongatus* BP-1 was followed. Having said that however, it must be highlighted that the

360-*fts*H ORF in particular, consisted the first priority in this study due to its strongest similarity score with the *slr*0228FtsH. More details however on distribution of FtsH proteins in cyanobacteria and their homologies are in chapter for Computational analysis of FtsH proteins.

	JGI gene ID **637798703**	JGI gene ID **637799360**	JGI gene ID **637799417**	JGI gene ID **637799745**
Locus Tag	Synpcc7942_0297	Synpcc7942_0942	Synpcc7942_0998	Synpcc7942_1314
Product Name	ATP-dependent metaloprotease	ATP-dependent metaloprotease	ATP-dependent metaloprotease	ATP-dependent metaloprotease
DNA coordinates / size	294872..296713 (+) / 1842 bp	947957..949849 (-) / 1893bp	1005621...100759 (+) / 1899bp	1342534...134445 (-) / 1872 bp
Amino acids sequence length	613 aa	630 aa	632 aa	623 aa
Transmembrane helices	Yes	Yes	Yes	Yes
Accession (NCBI)	YP_399316.1	YP_399959.1	YP_400015.1	YP_400331.1
External links	Gene ID: 3773859 UniProtKB: Q31RJ0	Gene ID: 3775329 UniProtKB: Q31PP7	GeneID: 3773925 UniProt/ TrEMBL: Q31PJ1	GeneID: 3775507 UniProt/ TrEMBL: Q31NM5

Table III.3. **Genes encoding FtsH proteins in *Synechococcus* 7942.** Along with the Gene ID used by the sequence Centre (JGI), there is additional information for each protein including, gene's size and location, and also gene and protein's IDs used by the most widely used Databases for ease of reference.

3.1. Deleting 360 *fts*H gene.

Following the same three step strategy a detailed plan was designed for interruption of 360*fts*H gene, Figure III.5. The large, PCR amplified fragment (1404 bp) of the ORF in question (1893bp) was disrupted with the Km^R cassette at the restriction sites of BmgBI and MscI endonucleases, leaving thus ~250 bp of 360*fts*H DNA sequence, flanking the antibiotic resistance ORF, for double cross-over recombination with the cyanobacterial chromosomal DNA.

For insertion of PCR band into MCS of pBluescript SK-, the restriction sites of XhoI and HindIII endonucleases were added to the forward and reverse primers respectively. To allow however cleavage close to the ends of the PCR product, four additional nucleotides were included at the 5' ends of each primer, Figure III.5.

Extracted from a dense culture, the chromosomal DNA was used as template for PCR amplification of 360 *fts*H ORF. Confirmed by AGE, the PCR band was then subjected to double digest with XhoI + HindIII, and when purified was set for ligation into linearised with the same set of endonucleases (XhoI + HindIII) cloning vector pBluescript SK. As both restriction enzymes XhoI and HindIII, produce cohesive ends which are not compatible, the linearised plasmid was not treated with Calf Intestinal Alkaline Phosphatase to avoid vector's re-circularisation.

The ligation mixture, set at 2:1 molar ratio of insert to plasmid DNA and left for 3 hours at ambient temperature, was then used to transform DH5*α* competent cells. Transformed *E. coli* cells, were subsequently planted onto LB agar plates containing the appropriate selective agents, namely ampicillin, and the chromogenic compounds X-gal and IPTG to allow the expression of blue & white colonies due to α-complementation. As described above, control plates were always set in parallel to the main transformation.

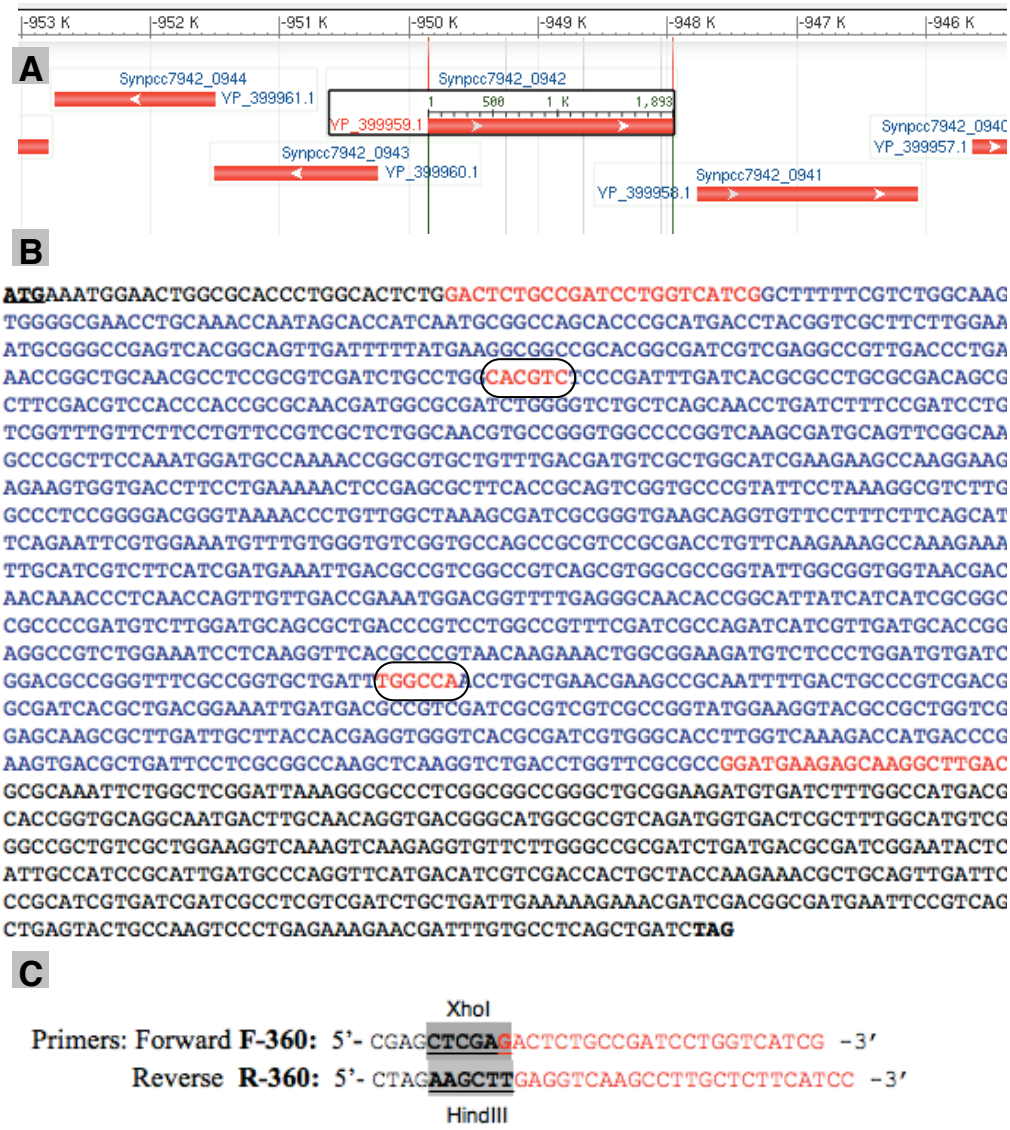


Figure III.5. **Design for disruption of 360 *fts*H ORF in *Synechococccus* 7942. A)** Location of the 360 *fts*H ORF in the chromosomal DNA. Complementary strand. **B)** DNA sequence of the ORF in question, where the band to be amplified by PCR is expressed in blue letters. The latter also includes the binding sites of the primers, depicted here in red, flanking the blue letters. Final PCR size with primers (binding site, restriction site and supplementary nucleotides) 1423 bp. Also in red, towards the middle of PCR band, and annotated with oval-shaped line are, from tom to bottom, the restriction sites of BmgBI and MscI endonucleases where the Km^R cassette will be cloned C). The sequences of the two primers. The highlighted-underlined letters correspond to the restriction sites of XhoI and HindIII. Letters with red denote the binding sites. The four additional nucleotides at the 5' prime ends in each primer are expressed with black letters.

Following "from colony to colony" molecular biology practice, recombinant plasmid, designated as pBSK-360 was obtained, Figure III.6. In particular, few, well shaped (~3mm in diameter), and clearly distanced white colonies were carefully picked and streaked out, for another run to prevent contamination, onto LB plates containing the

same selective agents as mentioned previously. Liquid cultures were then inoculated and used for plasmid DNA extraction.

Using different sets of restriction enzymes followed by agarose electrophoreses analysis the generated new recombinant plasmid was tested to verify the size and the cloned PCR fragment. Thus for instance a single digest with MscI, Figure III.6., confirmed not only the right size of the new plasmid, 4346 bp, but also the presence of this restriction site within the PCR band. An important confirmation as MscI, is one of the two restriction sites for inserting the Km^R cassette into 360*fts*H PCR fragment. Yet, the double digest with BmgBI + EcoRV mix, that cleaves the plasmid twice (Figure III.6) yielding two fragments of 3179bp and 1167bp, confirmed the expected orientation of the PCR band within the MCS and importantly the presence of BmgBI restriction site, that is part of the design (Figure III.5) for disrupting the 360*fts*H PCR fragment with Km^R

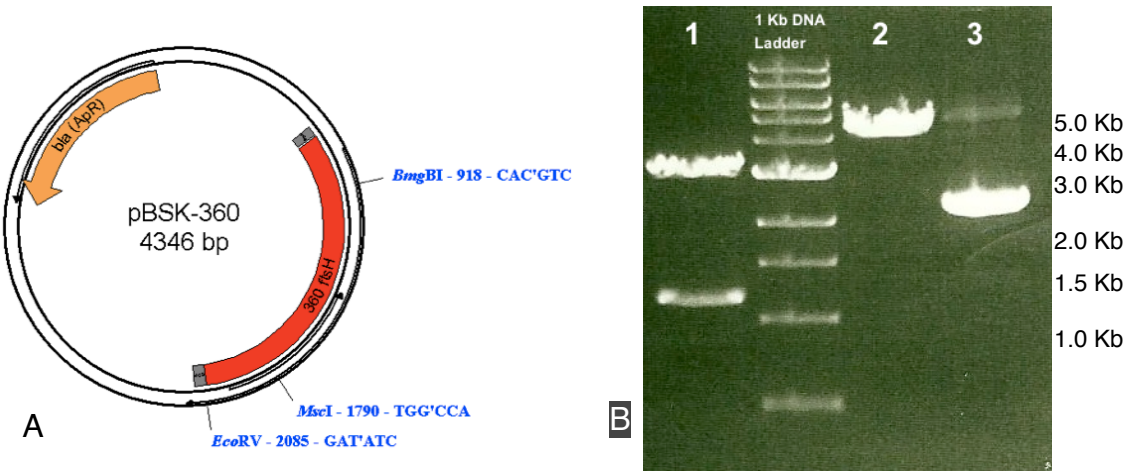


Figure III.6. **Recombinant plasmid pBSK-360 and restriction mapping.** A) Drawing of pBSK-360 (with pDRAW32 software) and the restriction sites of endonucleases: BmgBI; EcoRV; MscI. B) AGE Image of pBSK-360 digested with the above enzymes. In particular. Lane 1: Cleavage with BmgBI + EcoRV producing two bands of 3179bp +1167bp. Lane 2: Digest with MscI cleaving the plasmid once. Lane 3: pBSK-360 uncut.

The recombinant plasmid was sequentially digested (lack of compatible buffers) with BmgBI and MscI restriction enzymes (both blunt cutters), yielding two fragments of 872bp and 3.48Kb, with the former dismissed and the latter excised from the agarose gel. Thus, a large part of the 360ftsH gene, including almost the entire length of the AAA cassette was cut off .

For insertional disruption of the 360*fts*H ORF the kanamycin resistance cassette was used. To obtain it, the plasmid pUC4K was digested with HincII endonuclease (Figure III.3.), and the same procedures as described earlier in the case of *tll* 0734 were followed.

The concentrations of both DNA fragments, namely, truncated pBSK-0734 (now ~3.47Kb) and Km^R cassette were estimated, via agarose electrophoresis analysis. The molar ratio of fragment (Km^R) to plasmid DNA was set for 3:1 and the ligation was performed at room temperature for 20 hours. After transformation of competent *E. coli* DH5α cells with the ligation mixture, selection of recombinants cells relied utterly on combined resistance to antibiotics ampicillin and kanamycin, applied at concentrations of 100μg.ml^{-1} and 50μg.ml^{-1} respectively.

The putative plasmid, designed for interruption of 360*fts*H ORF in *Synechococcus* 7942 was extracted from overnight cultures of transformed *E.coli* cells, grown under the selective pressure of ampicillin and kanamycin combined. Prior to its use for mutation of our model cyanobacterium, the resulted new recombinant vector, henceforth referred to as pBSK-360 Km, Figure III.7., was subjected to detailed examination with several endonucleases and AGE analysis, Figure III.7.

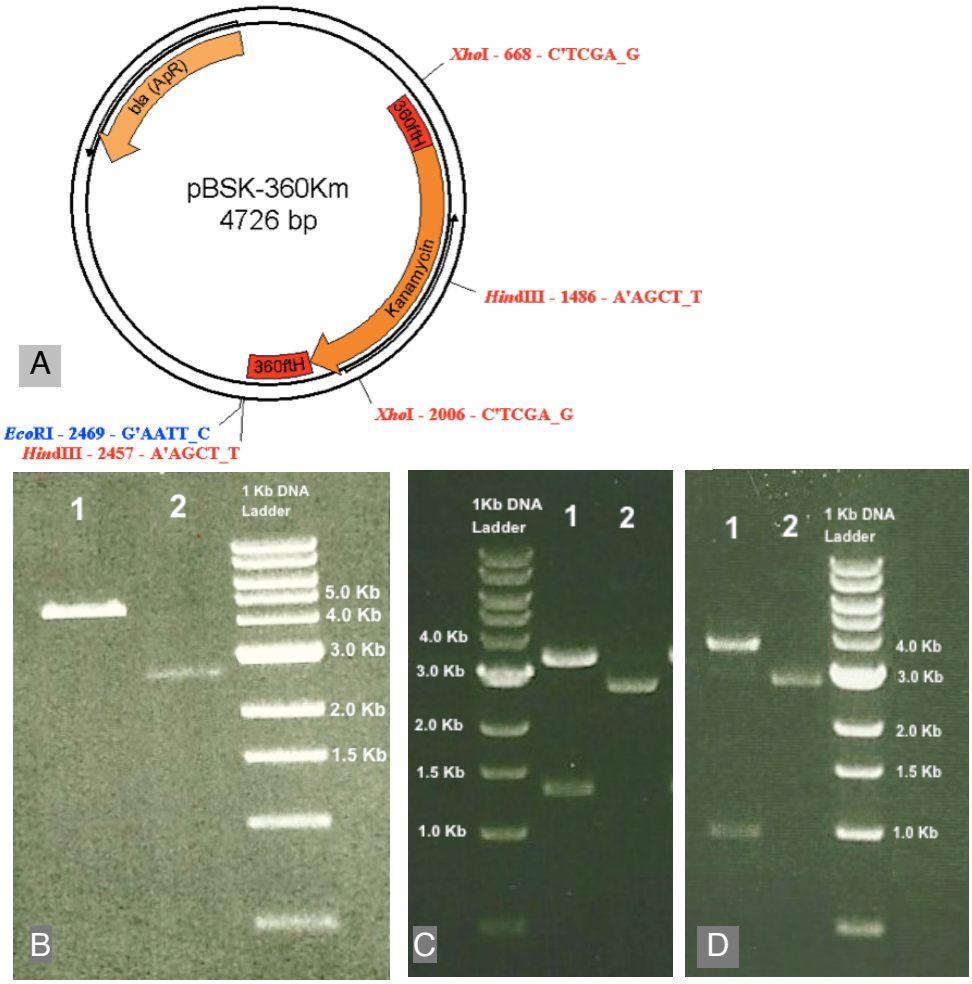


Figure III.7. **Plasmid pBSK-360Km and Restriction mapping.** A) Drawing of pBSK-360Km showing also the restriction sites of endonucleases used for plasmid's mapping. B) pBSK-360 Km cleaved EcoRI. Lane 1: digested plasmid. Lane 2: plasmid uncut. C) Digest with XhoI that cleaves the plasmid twice yielding two bands, 3388 bp + 1338 bp Lane 1. In Lane 2 - uncut plasmid. D) Lane1: pBSK-360 Km cleaved with HindIII yielding two bands 3755 bp and 971 bp. Lane 2 - plasmid uncut.

The new vector was analysed in detail using the enzymes XhoI, EcoRI, and HindIII. The final construct, referred to as pBSK-0734Km (size 4726bp), with fragments of 360FtsH, long enough (+240bp) for double recombination, flanking either side the kanamycin cassette, was subsequently used for transformation of wild type cells of *Synechococcus* sp. 7942.

3.2. Transforming *Synechococcus* 7942 with pBSK-360Km

After incubation at 30 ºC for four hours under dim light, transformed cells were spread onto BG11 agar plates and left to recover for 48 hours. Soft agar (0.6% w/v) containing the appropriate amount of kanamycin, was overlaid onto the transformation plates, which were incubated for 2-3 weeks at 30 ºC until green colonies appeared. Because

cyanobacterial strains under laboratory conditions may contain up to 10 copies of their chromosomal DNA, putative transformants were streaked out several times, providing thus sufficient time for complete segregation viz. disruption of the ORF in question in every copy of the chromosomal DNA.

To exclude the possibility of spontaneous mutations conferring resistance to antibiotic kanamycin, and above all to confirm the desired mutation of 360*fts*H, genomic DNA from wild type and mutated cells was used for PCR analysis. The results, Figure III.8., substantiated the mutation of 360*fts*H ORF, and yet, ruled out the presence of any residual copy(ies) of the unmutated gene. However, as several well shaped colonies of putative mutants were selected for further examination to confirm the mutation, after PCR and restriction analysis, only one colony henceforth was used as the source for mutant in the subsequent investigations.

With the disruption of 360*fts*H ORF confirmed, frozen stock of both, wild type and mutant cells (from the selected colony only) were prepared and stored at -70 ^{o}C for future experimental use. The mutant lacking the 360FtsH will be henceforth referred to as FtsH^{-} mutant.

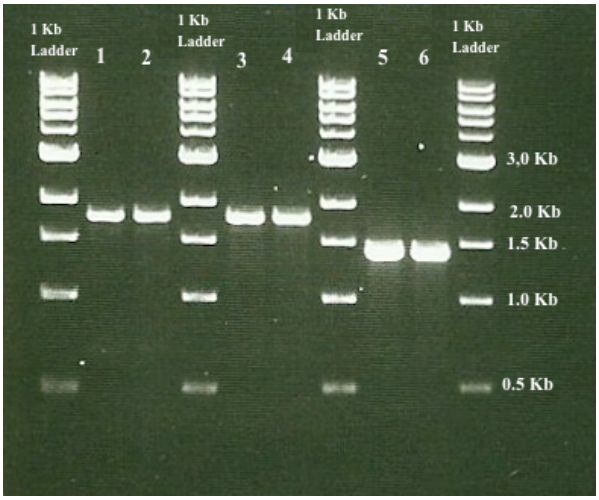


***Figure III.8*. PCR amplification of 360*fts*H ORF with DNA from *Synechococcus* 7942 wt and putative 360*fts*H less mutant cells.** DNA extracted from wt and putative mutant was used as template for PCR amplification of 360*fts*H ORF using the same set of primers (see Figure III.5) for both, wt and putative mutant. The PCR band from intact ORF in question is expected to be 1423 bp, whereas that of disrupted with the kanamycin cassette ~1789bp. Lane 1,2, 3,4 : PCR bands from putative mutants, derived from different colonies. Lane 5, 6: PCR bands from wild type cells.

4. Deleting 417*fts*H, 703*fts*H, and 745*fts*H ORFs in *Synechococcus* 7942

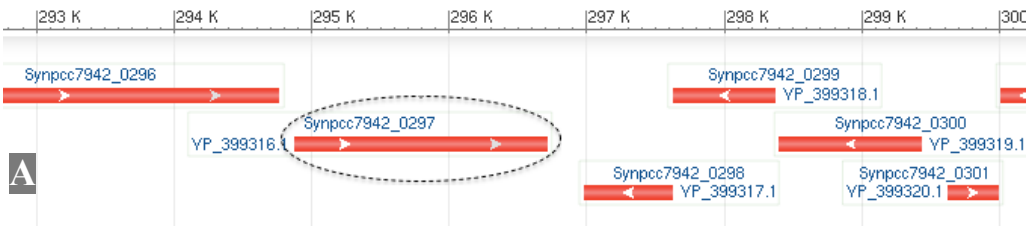
The disruption of the remaining *fts*H ORFs in *Synechococcus* 7942 was designed on the same three steps strategy of insertional mutagenesis. For the first step, namely, amplification of the targeted gene or large part of it by PCR and cloning it subsequently into the MCS of a suitable vector, the designed primers shared the same characteristic. In particular, after careful examination, it was found that the endonucleases SacI and XhoI cut none of the three targeted ORFs, and therefore their restriction sites were added, at the 5‘ ends, to the binding sites in each set of primers (Figure III.9., III.10., & III.11). Of course, to allow cleavage near the ends, four more complementary nucleotides were also included in each primer. The choice however of these particular endonucleases relied also on some attributes, favourable in molecular cloning practice. The cohesive ends produced by both enzymes, first facilitate the ligation and yet, require no additional restriction mapping to define the orientation of the ligated band within the plasmid. Needless to say that these two enzymes also cut the selected for the purpose vector pBluescript IIKS(-), within its MCS. Furthermore, the selected enzymes, do not produce compatible ends excluding thus the possibility of re-circularisation of the cloning vector and therefore of false transformations. Although SacI and XhoI can theoretically act simultaneously when in suitable buffer, in practice the sequential digest was preferred over the double, because the latter often led to incomplete digestion.

For the second step, viz., disruption of PCR fragment with an antibiotic resistance cassette, in contrast to the previous choices of kanamycin, three different antibiotics, chloramphenicol, spectinomycin and erythromycin were selected as selective markers. The decision for having other than kanamycin antibiotics, and different one for each *fts*H ORF was made exclusively on the possibility, organism permitted of course, for double or triple mutations.

4.1. Cloning *fts*H ORFs into pBluescript IIKS(-)

The same chromosomal DNA from previous applications was used in PCR amplification for the remaining *fts*H ORFs. The produced bands, 1429bp of 417 *fts*H; 1459bp of 703 *fts*H, and 1669bp of 745 *fts*H ORF, (Figure III.9., III.10. & III.11) after sequential digests with XhoI and SacI were subsequently cloned into linearised with the same enzymes pBluescript IIKS(-). All ligations were performed at room room temperature for four hours, with the molar ratio of plasmid to insert DNA set at 3:1, apart from that of 703 that was set at 2:1. Selection of the successfully transformed *E.*

coli cells, strains XL1-Blue and SCS-110 was once again based on *a*-complementation. Extracted from overnight cultures, the new recombinant plasmids, designated as: pBKSII-417; pBKSII-703; and pBKSII-745, Figure III.12., were then subjected to restriction mapping using several endonucleases such as XhoI, HincII, SacI, and others, Figure III.12. Control transformation were set in parallel as described above.



IMG Gene object ID 637798**703**

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TTGTCCAGCGCGTGGAAAACAAGCAAGTCGCTAAGGTTATCCTCAGTCCCGATCGCAGCTCG
GCACTGGTTCAAGCCGAAGACGGC**GACAAAGTGCAGGTCAATCTGC**CCAACGACCCGCAACT
GCTGAAAATCCTGACCGATAACAACGTCGATATCTCCGTCCGTCCTCAGAACCAAGACAGCG
TTTGGCTGCGGGCTCTGAGCAGCCTCTTCTTCCCGATCCTCTTGCTCGTGGGTCTGTTCTTC
ATCCTGCGCCGTGCCCAAGGTGGCCCTGGCAACCAGGCAATGAACTTCGGCAAGAGCAAAGC
TCGCGTCCAAATGGAGCCGCAAACCCAAGTCACCTTTAACGATGTGGCGGGGATTGATCAGG
CCAAACTGGAACTGACCGAAGTCGTCGACTTCCTCAAAAACGCCGATCGCTTCACCGCCGTC
GGTGCACAAATTCCCAAAGGCGTGCTGCTAGTGGGTCCTCCCGGTACCGGTAAAACCCTGTT
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TGGAAATGTTCGTCGGGGTCGGCGCTAGCCGCGTCCGCGACCTGTTCGAGCAAGCCAAAGCC
AGTGCGCCTTGCATCGTCTTCATCGATGAGATCGACGCCGTTGGTCGTCAGCGCGGTGCTGG
CCTCGGCGGTGGTAACGACGAGCGTGAGCAAACCCTCAACCAGTTGCTGACCGAAATGGATG
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GCGGCCTTGATGCGTCCCGGTCGTTTCGATCGCCAAGTGGTGGTCGATCGCCCCGACTACAA
CGGTCGTCTGGAAATCCTGCGCGTTCACGCTCGTGGCAAGAGCCTCAGCAAAGATATCGACC
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GAAGCCGCGATTCTGGCTGCTCGCCGCAGCTTGGCAGAAATCTCGATGGATGAAGTCAACGA
TGCGATCGATCGCGTCTTGGCGGGTCCTGAGAAGAAAGACCGCGTCATGAGCGAAAAACGCA
AAGTCTTGGTGGCTTACCACGAAGCTGGTCACGCCTTGGTCGGTGCCTTGATGCCTGACTAC
GACCCCGTCCAAAAAATCAGCATTATTCCCCGCGGCCGTGCGGGTGGCCTGACCTGGTTCAC
CCCCAGCGAAGAGCGGATGGAGTCGGGCCTCTACTCCCGTACCTACCTGCAAAACCAAATGG
CGGTTGCCCTTGGCGGTCGTCTGGCGGAAGAAATTGTCTTCGGTGAGGAAGAAGTGACGACT
GGCGCTTCTAACGACTTGCAGCAAGTCGCCCGCGTGGCTCGTCAGATGGTGACCCGCTTCGG
GATGAGCGATCGCCTTGGCCCTGTGGCCCTCGGTCGCCAGCAAGGCAACATGTTCCTCGGTC
GCGACATTGCTGCCGA**GCGTGACTTCTCGGAAGAGAC**TGCCGCCACGATTGACGACGAAGTG
CGTCAGCTGGTGGATGTTGCCTATGATCGCGCCAAGAAAGTGTTGATTGAAAACCGCTCGAT
TCTTGATCAACTTGCCAAAATGCTGGTTGAGAAAGAAACCGTTGATGCGGAAGAACTGCAAG
ATCTGCTCAACAACAATGAAGTGCGGATGGCCGCGATCGCC**TAA**

B

SacI

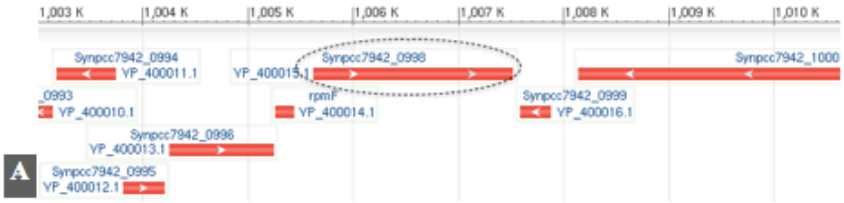
Forward : TCTA-GAGCTC -GACAAAGTGCAGGTCAATCTGC

Reverse: AGTA-CTCGAG- GTCTCTTCCGAGAAGTCACGC

XhoI

C

Figure III.9. Design for the disruption of 703*fts*H ORF in *Synechococcus* 7942. A) Location of the 703 *fts*H ORF in the chromosomal DNA. B) DNA sequence of 703*fts*H ORF. The band to be amplified by PCR is expressed in blue letters. The latter also includes the binding sites of the primers, depicted here in red. Nevertheless as the two primers also include the restriction sites of two enzymes and four more additional nucleotides at their 5' ends, the final PCR product is expected to be 1459 bp. Also in red but with grey highlighted background are the restriction sites of HincII for cloning of Chloramphenicol Resistance cassette, Cm^R. C). The sequences of the two primers. The highlighted letters correspond to the restriction sites of XhoI and SacI. Letters in red denote the binding sites. The four additional nucleotides at the 5' prime ends in each primer, are expressed with black letters



IMG Gene object ID 637799**417**

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CACACCGACACAGGACAACAGCGCCTTAGCAGGCACGCTGGTCAACCTCGGGCTGATTTTGATCCTGATCGTCGGCCTGG
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CGAGCTGATGGATCGACTCGTCGATCGCTTGATCGATCAAGAACTGATTGAGGGCGATGAATTTCGCAAAATTGTTGAAC
AATTTCCGAAATCGAGTGCAGTGACTCAGCCAGCGATCCAAGCACCCGCTGTGTTT**TAG**

B

SacI
Forward: TCAT-GAGCTC-GACAGTCTCCCTCGATTACTCG
Reverse: AGTA-CTCGAG-CGATGTCAGAAGCGGCACC
XhoI

C

Figure.III.10. **Design for disruption of 417 *fts*H ORF in *Synechococcus* 7942.** A) Location of the 417 *fts*H ORF in the chromosomal DNA. B) Sequence of the entire 417*fts*H ORF. Blue letters - the fragment to be amplified by PCR, which also includes the binding sites of the primers, depicted here in red. Final PCR size, including the restriction sites of two enzymes and four more additional nucleotides at their 5' ends, is 1429 bp. Also in red but with grey-highlighted background are the restriction sites of HincII and EagI endonucleases for cloning of Sp^R - Spectinomycin resistance cassette C). The sequences of the two primers. In red: the binding sites. Highlighted: the restriction sites of SacI & XhoI. Finally, the four nucleotides at 5' ends, shown in black letters, are supplementary to allow cleavage close to ends.

IMG Gene object ID 637799**745** 1872bp, PCR 1649bp

ATGGCCATCAAGGAGAATCCCACGCGGCCGAGCCGCAACCGTATTATCAGCTTCGTCCTGTTTGGTGTGGGTCTGTTCTT
TTTGCTGATTGGGTTAATTCCCGGCGTGGGCAGTCCCCAAGTCTCTCGGGTGCCCTACAGCCTATTCATCGATCAGGTGA
ATGATGGCTTGGTTGCCCGGGCTTACATCACGCAGGAACAAATTCGCTACCAACTCAAGGATGTTGAGGGCGAAGCGGGT
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GGTGATGATACCCGTACCACCTTCAGTGATGTGGCGGGCGTCGAAGAGGCGAAGGCCGAACTGCAAGAGATTGTTGACTT
CCTCAAAACCCCTGAACGCTACCTCAACATTGGCGCTCGTATTCCAAAAGGGGTATTGCTGGTCGGGCCTCCGGGTACCG
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ACACTGGATCCGGCATTACTACGTCCGGGTCGCTTCGATCGCCAAGTCCTAGTCGATCGCCCTGATCTGGCGGGACGGAA
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ACGGATTGTTGCCTACCACGAAGTCGGCCACGCGATCGTGGGTGCCCTGATGCCCGGTGGCAGCAAAGTGGCCAAGATTT
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GAACTCAAAGGCCAGATTGCCACGCTGCTAGGTGGGCGATCGGCGGAAGAGATCATCTTTGGCAGCATCACCACCGGCGC
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GCGAAGGCGATCGACGCAGAGGTCAAGCAACTCGTGGATGATGGTCACGATCAAGCCCTGGCAATTCTGAATCGCAATCG
CGATCTACTCGAGGAAATTGCTCAGCGCATCTTGGATGTGGAAGTGATTGAAGGCGACGAGCTGCAATCCCTCCTCCAAC
GCCCGAGTTGCAGCCAGCC**TAA**

B

XhoI
Forward: AGAT-CTCGAG -CTACAGCCTATTCATCGATCAGG
Reverse: TCAT -GAGCTC-GAGCAATTTCCTCGAGTAGATCG
SacI

C

Figure.III.11. **Design for disruption of 745 *fts*H ORF in *Synechococcus* 7942.** A) Location of the 745 *fts*H ORF in the chromosomal DNA. B) The sequence of 745*fts*H ORF, with the fragment to be amplified by PCR expressed in blue letters. The final PCR product, also including the binding sites of the primers, depicted here in red, the restriction sites of XhoI and SacI and the 4 supplementary nucleotides is expected to be 1669bp. Also in red but annotated with grey-highlighted background are, from top to bottom, the sequences of the HincII and MscI endonucleases for cloning of Erythromycin resistance cassette (Er^R). C). The sequences of the two primers. The highlighted letters correspond to the restriction sites of XhoI and SacII. Letters in red denote the binding sites of the primers. The four additional nucleotides at the 5' prime ends in each primer are expressed with black letters

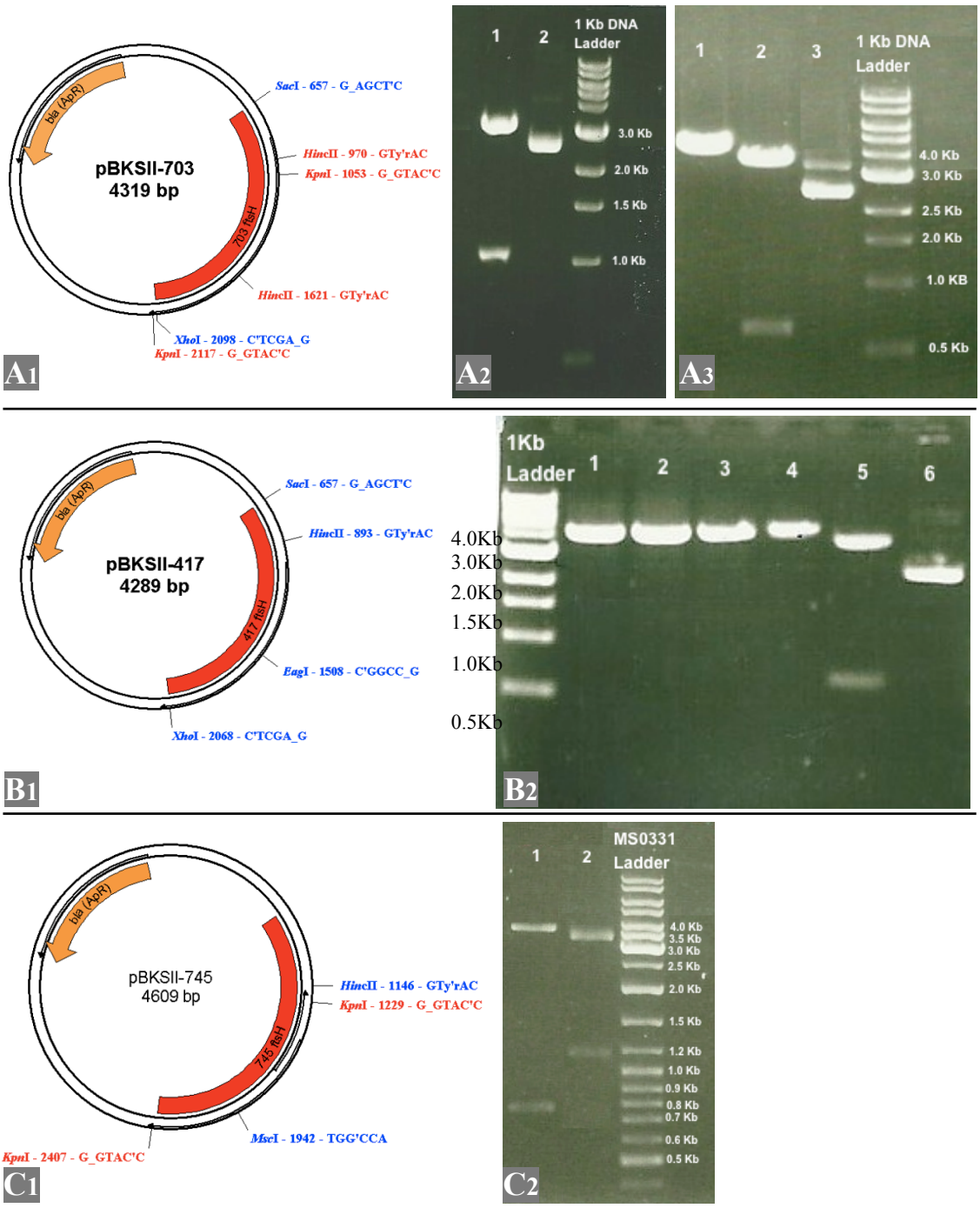

Figure III.12. **Recombinant plasmids containing parts *fts*H ORFs.** A1) pBKSII-703 vector plasmid a A2- In Lane1: digest with KpnI. Lane 2: uncut plasmid. A3 - Lane 1: linearised with SacI. Lane2: cleaved with HincII (~3.7 & 0.7 Kb). Lane3: uncut plasmid. B1) pBKSII-417, B2- Lane1: cleaved with SacI. Lane2- with XhoI. Lane 3- with HincII. Lane4: with EagI. Lane5: with HincII+EagI. Lane 6- uncut plasmid. C1) pBKSII-745. C2 - Lane1: cleaved with HincII and MscI. Lane2: with KpnI

4.2. Disrupting fragments of *fts*H ORFs with antibiotic resistance cassettes

For the second step of mutagenesis strategy, namely disruption of *fts*H ORF fragment, in pBKSII-703, pBKSII-417 and pBKSII-745, with a drug resistance cassette, genes conferring resistance to chloramphenicol. spectinomycin and erythromycin were respectively used. More details about this step are in Table III.4.

	Vector plasmids for insertional mutagenesis of *fts*H ORFs		
	pBKSII-703Cm	**pBKSII-417Sp**	**pBKSII-745Er**
Plasmid to be disrupted (size)	pBKSII-703 (4.3 Kb)	pBKSII-417 (4.3Kb)	pBKSII-745 (4.6Kb)
• *Enzymes used*	• HincII	• EagI + HincII	• HincII + MscI
• *Produced bands*	• 3.7 Kb + 0.7 Kb	• 3.7 Kb + 0.6 Kb	• 3.8 Kb + 0.8 Kb
Antibiotic Resistance Cassette (Symbol)	Chloramphenicol (CmR)	Spectinomycin (SpR)	Erythromycin (ErR)
• *Derived from*	• pUC4Cm	• pBsSp1.1	• pBsEmV
• *Enzymes used*	• HincII	• EagI & HincII	• EcoRV
• *Cassette's size*	• 0.84 Kb	• 1.1 Kb	• 1.45 Kb

Table III.4. **Vector plasmids used for insertional mutagenesis of *fts*H ORFs in *Synechococcus* 7942.**

The chloramphenicol resistance cassette used to disrupt pBKSII-703, was produced from pUC4Cm plasmid using HincII endonuclease. The same enzyme cuts pBKSII-703 twice, both within the 703*fts*H fragment. The large band ~3.7Kb was used in the next step application, while the small band, ~0.7Kb, dismissed. Excised from an Agarose Gel, both the Cm^R and the ~3.7Kb fragment from pBKSII-703, were set for ligation at 16 ^oC for 20 hours and at molar ratio of insert to plasmid DNA to be at 4:1. Competent XL-1 Blue cells were transformed with the ligation mixture and spread onto selective LB agar plates containing the antibiotics ampicillin and chloramphenicol at concentrations 50μg.ml^{-1} and 10μg.ml^{-1} respectively. Recombinants were picked from well shaped (2-3mm) colonies, and after inoculation of liquid cultures, the final desired construct pBKSII-703Cm (4.5Kb) was produced, Figure III.13. Although large part (~0.7Kb) of 703*fts*H fragment was dismissed with the insertion of Cm^R, the fragments

of *fts*H gene left flanking the drug cassette were long enough (+300bp and +450bp) to allow double homologous recombination with the cyanobacterial chromosomal DNA.

The spectinomycin resistance cassette was used to interrupt the DNA sequence of the 417*fts*h ORF in the recombinant plasmid pBKSII-417. The Sp^R was excised from an agarose gel after cleaving the plasmid pBsSp1.1. sequentially with HincII and EagI endonucleases. The same set of enzymes, was used to digest pBKSII-417. Excised also from agarose gel, the truncated pBKSII-417 plasmid, ~3.5Kb , and the spectinomycin cassette were mixed at molar ration 1:4 respectively and left to ligate at ambient temperature for 16hours. Transformed *E. coli* competent cells, strain XL-1 Blue, were selected on LB agar plates containing antibiotics ampicillin and spectinomycin at concentrations 50µg.ml^{-1} and 30µg.ml^{-1} respectively. The final construct pBKSII-417Sp (~4.6KB) was extracted from selected recombinants and before being used for transformation of wild type *Synechococcus* 7942 cells, was subjected for restriction analysis, Figure III.13.

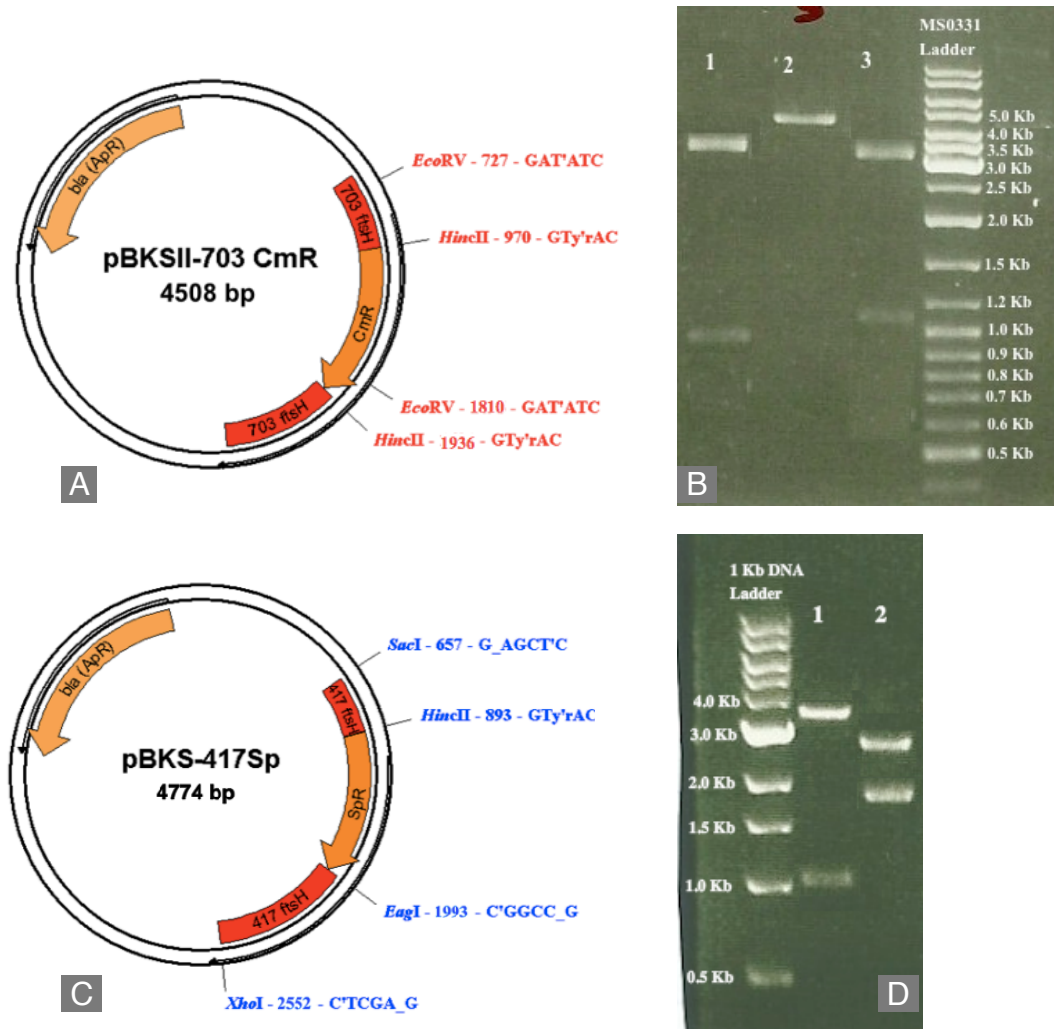

Figure III.13. **Recombinant plasmids pBKSII-703 & pBKSII-417, and their restriction analysis**. A) Drawing of vector plasmid pBKSII-703Cm with restriction sites of EcoRV and HincII. Each enzyme cuts the plasmid twice producing two bands B) AGE image from restriction analysis of the pBKSII-703Cm. Lane1: digest with HincII producing two bands 966bp + 3542bp. Lane2: plasmid linearised. Lane 3: digest with EcoRV producing also two bands 1083bp + 3425bp. C) Drawing of vector plasmid pBKSII-417Sp with the restriction sites of SacI, HincII, EagI, and XhoI. D) Restriction analysis of pBKSII-417Sp. Lane1: plasmid cut with SacI +XhoI produces two bands 1895bp + 2879bp. Lane2: plasmid cut with HincII + EagI. Two bands produced 1100bp + 3674bp

For disruption of 745*fts*H ORF in pBKSII-745 vector plasmid, the Erythromycin resistance (Er^R) cassette was used. To obtain the latter, the plasmid pBsEmV, bearing the cassette in question, was cleaved with EcoRV endonuclease producing blunt ends. The plasmid to be disrupted, pBKSII-745, was digested with MscI and HincII, both producing blunt ends. Despite numerous attempts and with various settings of molar ratio and temperature, any endeavour for the above ligation met with no success. Use of

commercial, super-competent cells, strains SCS-110 and XL1-Blue led to some peculiar results. Although the transformed with the ligation mixture cells were sometimes able to grow on LB plates containing both antibiotics viz., ampicillin and erythromycin, any attempt to inoculate liquid cultures for plasmid extraction, using the same selective pressure (ampicillin and erythromycin) was ultimately unsuccessful. Growth, either on or in LB medium was only possible with one of the antibiotics present but not both of them. Yet, no plasmid DNA was extracted from the liquid cultures grown in media with one antibiotic. For that reason, and also that this particular ORF shows the highest similarity to *slr*1463 in *Synechocystis* 6803, deletion of which had no obvious phenotype under laboratory conditions, (Mann *et al.,* 2000) this project was finally abandoned.

5. Results

Despite the numerous attempts, mutagenesis of 703 and 417 *fts*H genes in *Synechococcus* sp. 7942 met with no success. These seemingly negative results however merit a different, contrasting interpretation. Given the successful mutation of 360-*fts*H ORF and the use of the same protocol and materials in every transformation attempt, unsuccessful mutagenesis of 703 & 417 *fts*H-genes may be due the significance these particular genes bear for cell homeostasis and survival. This proposal correlates well with mutagenesis studies in *Synechocystis* sp. 6803 where clones carrying mutations in ORFs *slr*1390 and *sll*1604 failed to segregate even after multiple rounds of re-streaking, suggesting thus the prime importance of these two FtsH proteases for cell viability (Mann *et al*., 2000). Yet in the chapter on computational analysis of FtsH proteins, it has been demonstrated that the orthologs of *sll*1604 & *slr*1390 in *Synechococcus* 7942 are precisely these two FtsHases viz., 703 and 417, proved to be too important for the cell to have them deleted.

CHAPTER IV

CHARACTERIZATION OF 360FtsH-less MUTANT IN *SYNECHOCOCCUS* PCC7942

Chapter IV

Characterization of 360FtsH-less mutant in *Synechococcus* PCC 7942

1. Introduction

The multisubunit complex of Photosystem-II, of which the repair cycle is subject to investigation in the current project, has two unique features setting it apart from any other known to date units operating in photosynthetic organisms.

First, its uniqueness lies in the fact that it drives one of the most, if not the most thermodynamically demanding reactions known to occur in nature, namely the splitting of water. The other distinct feature of this complex is that it turns over more frequently than any other membrane embedded structure involved in photosynthesis (Barber and Anderson 1992; Prasil *et al.,* 1992. Aro *et al.,* 1993; Adir *et al.,* 2003) Due to its highly oxidising regime, required to oxidise water, this multisubunit complex is being subjected to irreversible damage at all light intensities (Barber & Anderson 1992, Prasil *et al.,* 1992, Aro *et al.,* 1993, Edelman & Mattoo 2008, Nixon *et al.,* 2005), thus falling victim of its own success if stated in more pragmatic terms. Among its numerous structural subunits, only one, and in particular the D1 core protein, that binds the majority of co-factors involved in electron transfer, almost inevitably becomes the main target of this light-induced damage (Barber & Anderson 1992, Nixon *et al.,* 2005; Edelman & Mattoo 2008).

To counteract this problem, photosynthetic organisms have evolved a mechanism repairing the impaired PS-II by replacing the damaged D1 subunit with a newly synthesised copy (Edelman and Matto 2008; Nixon *et al.,* 2005; 2010). For a good number of reasons, based on experimental data, it is believed that prior to its replacement, the D1 subunit undergoes degradation of proteolytical nature and some members of FtsH proteases play an essential role in the early stages of this process (reviewed by Nixon *et al.,* 2010).

Availability of the genome sequence of *Thermosyncechoccosus elongatus* BP-1, and then of *Synechoccocus* 7942 led us to investigate the importance of FtsH proteases in the Repair Cycle of PS-II. Given that the species of *Synechoccocus* genus constitute a large and diverse group of organisms inhabiting fresh waters

and practically all marine environments from poles to tropics (Stockner *et al.,* 2000, Ferris & Palenik. 1998; Passoni & Callieri 2001; Wetzel *1989*), understanding the role of FtsHases in these organisms might be essential to understand their role in cyanobacteria in general.

The 360*fts*H gene in *Synechococcus* 7942 was targeted for insertional inactivation and the resulted mutant was subjected to investigation having always as reference the respective characteristics of the wild type cells. Along with various facets of the mutant examined in this study, described in details in the following chapter, the first step in characterising the mutant was to understand the overall impact of *360fts*H deletion on cell's operation and appearance, e.g., growth and pigmentation.

2. Response to light

Almost inevitably, as this project was in the great field of photosynthesis, the overall characterisation of the mutant involved light and was largely if not exclusively focused on those parameters that to one or another degree take part and facilitate the photosynthetic activity of the organism.

Considering the impact that the light intensity has on the performance of the photosynthetic apparatus and D1 turnover (Aro *et al.,* 1993; Barber & Anderson 1992; Nixon *et al.,* 2005), to acquire a more comprehensive picture of the functional implications of 360FtsH protein, both types of cells were grown under different light intensities.

Thus, in an attempt to portray the overall picture and reactions of the mutant, always collated with the wild type, the growth rate along with the pigment composition during growth, were recorded using room temperature whole cell absorption spectra.

2.1. Growing under normal light conditions

Both type of cells were grown under standard incubatory conditions under constant illumination of white fluorescent light, with the light intensity adjusted to 10 μmol. $m^{-2}.s^{-1}$. For consistency, the whole cell absorption spectra were taken at equal time intervals, every 24 hours.

2.1.1. Growth Rate

As bacterial cell suspensions scatter light in proportion to their numbers, the growth rate was calculated by measuring the optical density OD at 750nm of 1ml sample of cells or a suitable dilution at later stages. The readings were recorded once a day at the same time and for 16 days.

From the constructed curves (Figure IV.1) emerged that the lag phase for the wt and FtsH$^-$ mutant continued for 4-5 days, during which the growth rate was slow and similar for both types of cells, albeit slightly faster for the wt.

However, as the cells entered into an apparent fast-paced phase of growth (5-6th day onwards), the wt exhibited noticeably faster rate compared to that of FtsH$^-$ mutant (Figure IV.1).

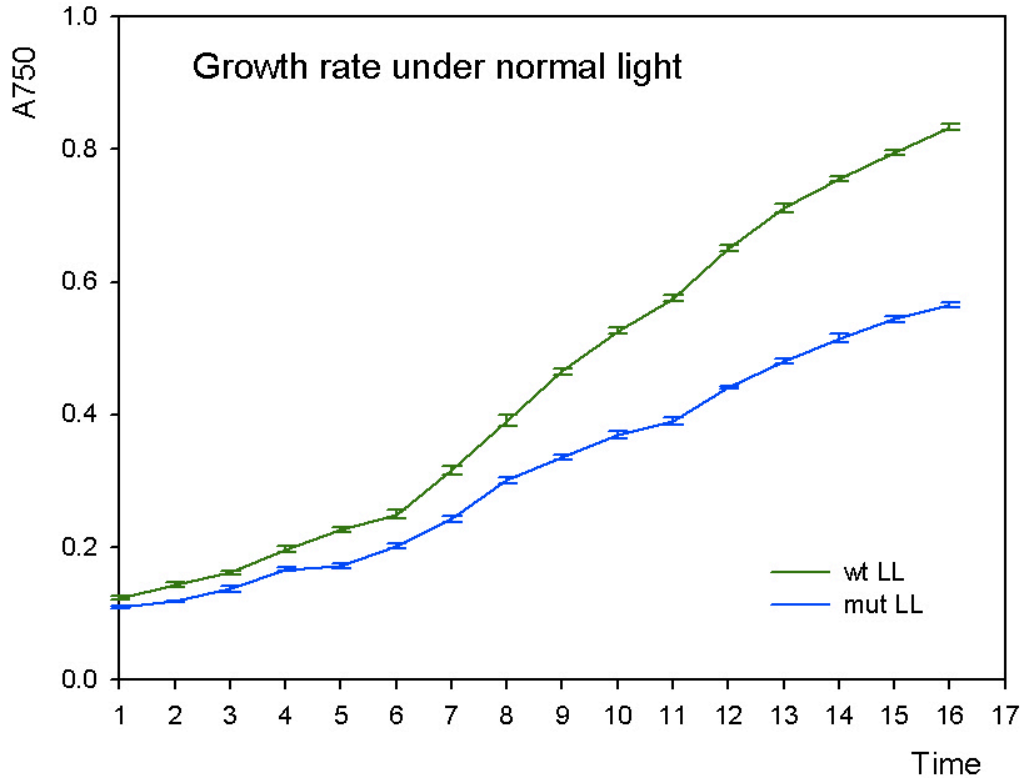

Figure IV.1. **The growth rate of wt and FtsH$^-$ less mutant, grown under constant illumination of 10 µmol. m^{-2}. s^{-1}., of white fluorescent light.** Time, is expressed in days. Number one corresponds to the day of inoculation. The values in this graph are the averages of at least three independent experiments (replicants), and the error bars denote the standard error.

2.1.2. Pigmentation

As FtsH has been reported to be involved in the assembly of functional PS-I (Mann *et al.,* 2000), and also in the early stages of the D1 degradation in *Synechocystis* sp 6803 (Silva *et al., 2003;* Komenda *et al.*, 2006), the pigment content per cell was an interesting and important factor to investigate and quantify as well.

To calculate the concentration of chlorophyll-*α* and phycocyanin (PC) in intact cells, the absorbances at 750nm, 678nm and 625nm were recorded and used in the formulas of Myers *et al.,* 1980 (Chapter II).

However, given that calculation of chlorophyll and phycocyanin concentrations were initially expressed per volume of culture (mM), while it is of a greater value to know the number of molecules of these pigments per cell, to obtain the latter, the number of cells per volume of culture were also calculated.

The pigment content in each type of cells

Wild Type

Growing under normal light conditions the pigment content (chlorophyll-*α* & PC) in wt cells remained largely unchanged throughout the experiment (Figure IV.2 & 3). During the course of 16 days, the mean cellular contents of chlorophyll-*α* and PC molecules were calculated to be $8.6*10^7$ and $5.7*10^7$ respectively while the PC/Chl ratio remained steady at 0.68 (Table IV.1).

However, plotting the concentrations of chlorophylls and phycocyanins per cell against time (Figure IV.3), disclosed a new pattern or tendency in pigments concentrations during their growth a feature that can not be reflected by mere calculation of the mean concentrations. From the constructed graphs (Figure IV. 3) emerges that as the cells enter into an accelerated stage of their growth (4-5th day onwards), the pigment content per cell, as of chlorophyll as of phycocyanin, seems to drop slightly for as long as the cells are dividing regularly and are going through the fast-paced growth rate. The level of concentration for both pigments is restored as the cells enter into the stationary phase.

FtsH-less mutant.

Growing under the same conditions as the wt, the $FtsH^-$ mutant exhibited nearly the same patterns in pigments composition. The pigment content in mutant cells, with minor fluctuations remained unchanged throughout the observation time (Figure IV.2 & 3) and the mean content of molecules per cell for chlorophyll-*α* and PC was: $6.7* 10^7$; $5.4* 10^7$ respectively, while the PC/Chl ratio was calculated to be 0.83 (For details see Table IV.1)

	wt	**$FtsH^-$ mutant**
Chlorophylls/ cell	$8.6 *10^7$ ± $2.1*10^6$	$6.7* 10^7$ ± $1.4* 10^6$
Phycocyanin/ cell	$5.7*10^7$ ± $1.29* 10^6$	$5.4* 10^7$ ± $6.9* 10^6$
PC / Chl	0.68 ± 0.01	0.83 ± 0.03

Table IV.1. **Mean number of pigments (chlorophyll & phycocyanin) per cell in wt and $FtsH^-$ mutant *Synechoccocus sp* 7942**. The mean values are the result of at least 40 measurements.

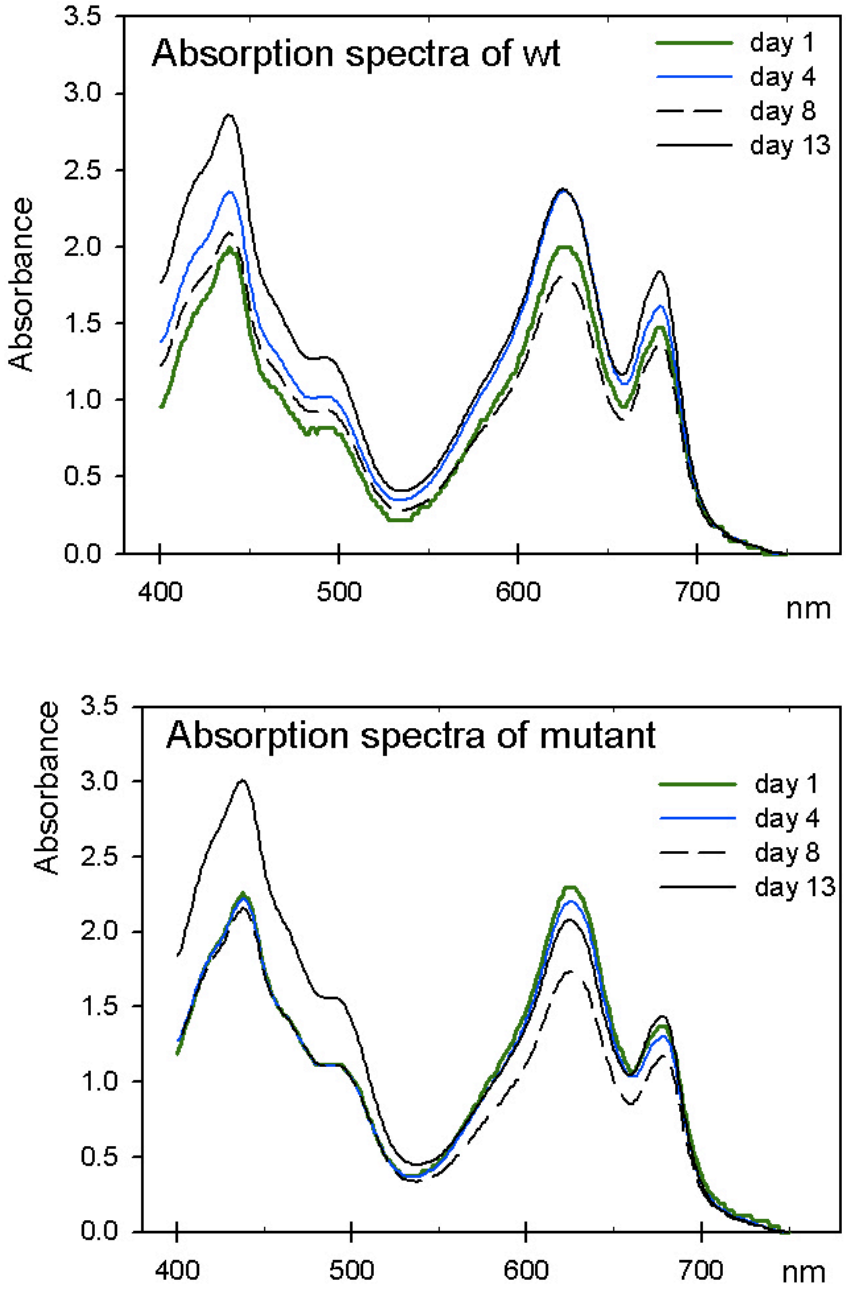

Figure IV.2 . **Whole cell Absorption Spectra of wt and FtsH$^-$ mutant.** Solely for clarity reasons, only four graphs out of 16 are expressed here that are nonetheless sufficient to illustrate the fact that the pigment composition in cells stayed largely unchanged during the course of sixteen days.

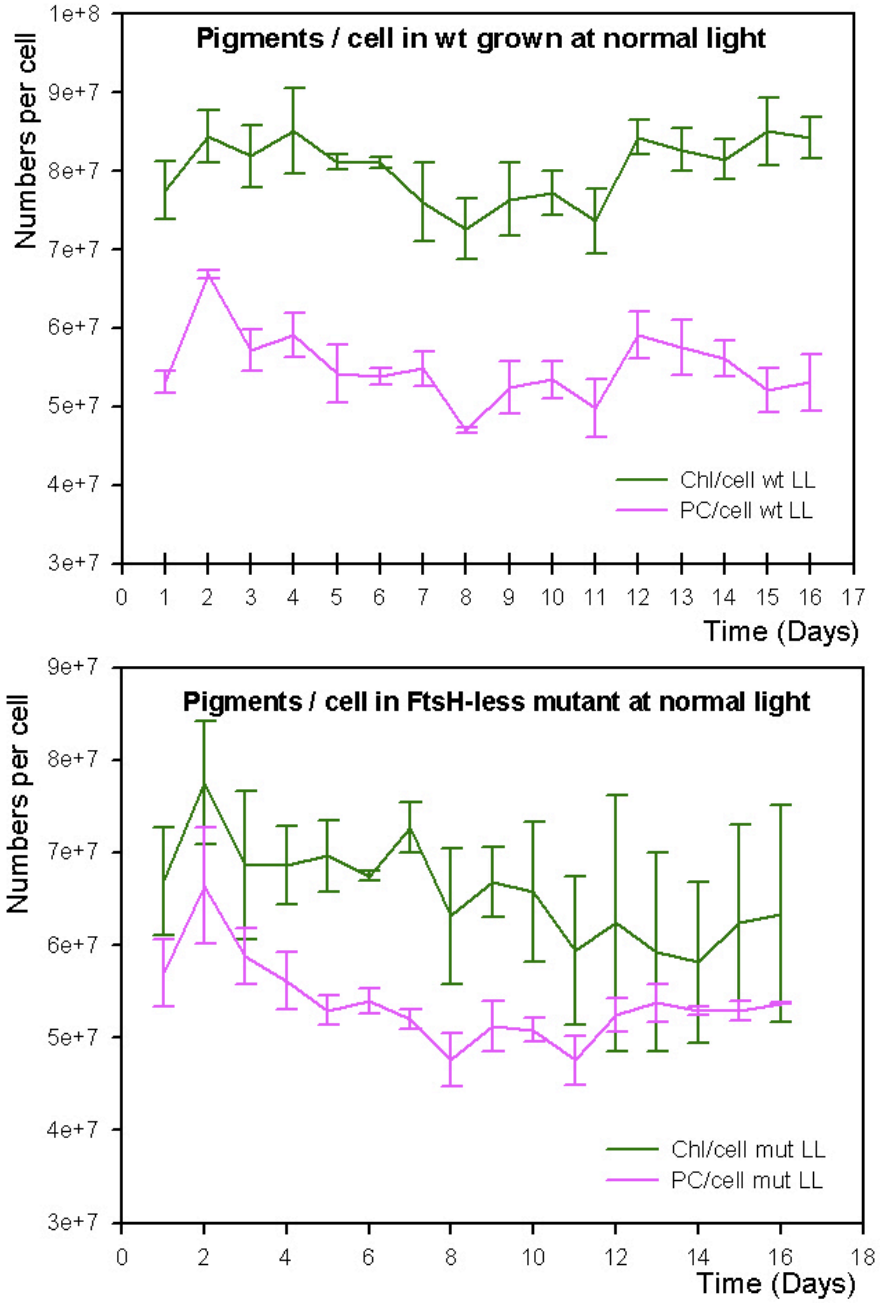

Figure IV.3. **Concentration of Chlorophyll and Phycocyanin during growth.** Calculated cellular concentration of two pigments are plotted against time, expressed in days. Number one refers to the day of inoculation. The mean values are the averages of at least three replicants, while the error bars correspond to standard error.

2.1.3. Differences between the two types of cells

Grown under normal light conditions, both types of investigated cells in the present project, exhibited similar patterns of growth and in pigmentation while at the same time they also appeared to have some noticeable differences.

As far as the growth rate is concerned, both types appeared to require the same adaptation period (lag phase) and both enter into the fast growth stage simultaneously. Apart from these similarities however, there is an evident difference, that the wt cells exhibit a tangibly faster growth rate than the FtsH$^-$ mutant.

The study of pigment composition revealed that the overall content of pigments in each type of cells remained nearly unchanged (Figure IV.2 & 3 and Table IV. 1). Illustrative of this is the PC/Chl ratio which stayed characteristically steady throughout the duration of the observations and in high contrast when cells were grown under High Light conditions (for details see below).

However, when the data from the spectroscopy for these two types of cells were analysed and compared in between them, they showed some significant differences. The first dissimilarity, although only visual, was the coloration of the cultures as the cell suspensions of FtsH$^-$ mutant appeared to be slightly more bluish rather than blue-green as that of the wt. With the content of phycocyanin almost the same in both types, the difference in colour seem to come from the amount of chlorophylls in each type of cells (Table IV.1). In FtsH$^-$ mutant it is significantly lower, only 80% of that in the wt strain, hence the notable increase in PC/Chl ratio in FtsH$^-$ mutant. The results correlate well with the findings reported in *Mann et al., 2000,* where the whole cell absorption spectra showed a clear increase in phycocyanin/chlorophyll ratio in slr0228FtsH-less mutant of *Synechocystis* 6803.

As mentioned above the wt cells of *Synechoccocus* 7942 show a slight tendency to reduce their pigment content during the stage of accelerated growth that however returns to its original level during the stationary phase. This particular trend though, is not evident in the cells carrying the mutation (Figure IV.3)

2.2. Growing under High Light Conditions

It is known that the PS-II D1 core protein is being damaged constantly under any light, and most importantly, this damage is a linear function of the intensity

of light (Aro *et al.,* 1993, Barber & Anderson 1992; Nixon *et al.,* 2005). As this course project is about a particular FtsH that has been shown in other organisms to be involved in the assembly of functional PS-I (Mann *et al.,* 2000) and in the proteolytical degradation of the D1 protein (reviewed by Nixon *et al.,* 2010), we wanted to examine the phenotype of cells lacking this protease at high irradiance when the Repair Cycle is tested more critically.

Exposing the cells to intense light correlates with the ecology of freshwater *Synechoccocus* species, where our model organism derives from (Wetzel 1989). Freshwater *Synechococcus* strains are highly plastic and have been found to thrive in both surface and deep waters (Stockner *et al.,* 2000) and in lakes can acclimate to different environmental conditions and irradiance levels by adjusting their pigment composition (Hauschild *et al.,* 1991; Vörös *et al.,* 1998; Callieri *et al.,* 2007)

Examination of the overall phenotype of the mutant versus that of wt strain when cultured under normal light conditions (10 μmol. $m^{-2}.s^{-1}$) showed few but nonetheless distinct differences between them. In contrast, continuous illumination of cells with 160 μmol. $m^{-2}.s^{-1}$ triggered different responses in each type of cells and clear, plain evidence appeared from the beginning of the experiment.

For consistency, identical conditions of growth were used as when the cells were grown under normal light condition but with a sole difference the intensity of light this time to be adjusted at 160 μmol. $m^{-2}.s^{-1}$. Again for regularity the same equipment, settings, methods and formulas were used as above and the measurements were taken every 24 hours at equal intervals.

2.2.1. Growth Rate

Illumination of cells with intensity as high as 160 μmol. $m^{-2}.s^{-1}$ resulted not only in significant increase of growth rate for each type of cells but also produced some unexpected results.

The wt and the $FtsH^{-}$ mutant responded to high light with substantial increase of growth rate (Figure IV.4) compared to that under 10 μmol. $m^{-2}.s^{-1}$, suggesting that the limiting factor for the relatively slow growth in the latter case was the

quantity of light. With a short lag phase both types of cells, evidentially, start dividing more rapidly only 24 hours or less after the inoculation.

Incubation of cells under high light produced also some unexpected results, a shift in growth pace from the fourth day onwards, Figure IV.4. The rate of FtsH$^-$ mutant is faster than that of the wt. Studying the graphs in Figure IV.4 more carefully and taking into consideration the findings about the pigment composition in our model cells, it is more accurate to state that the rate of growth of the wt cells declines after the third day while that of the FtsH$^-$ mutant remains unchanged and thereby appears to be faster.

Explanation of the aforementioned phenomenon is difficult. FtsHases are evidently involved in numerous cellular activities of different nature, from chaperone-like to proteolytic ones. In *E. coli* they are one of the most significant regulatory proteases involved in membrane biogenesis (Ogura *et al.,* 1999). They do not only control the quality of membrane proteins by degrading any abnormal forms, but above all balance the biosynthesis of major membrane components, i.e., phospholipids and lipopolysaccharides (LPS) which are mutually competitive (Ogura *et al.,* 1999). Because of the complex interactions in these multistep biochemical pathways, e.g. synthesis of phospholipids and LPS, disruption of an enzyme that is engaged in any one step during these processes, might affect other characteristics of the overall phenotype.

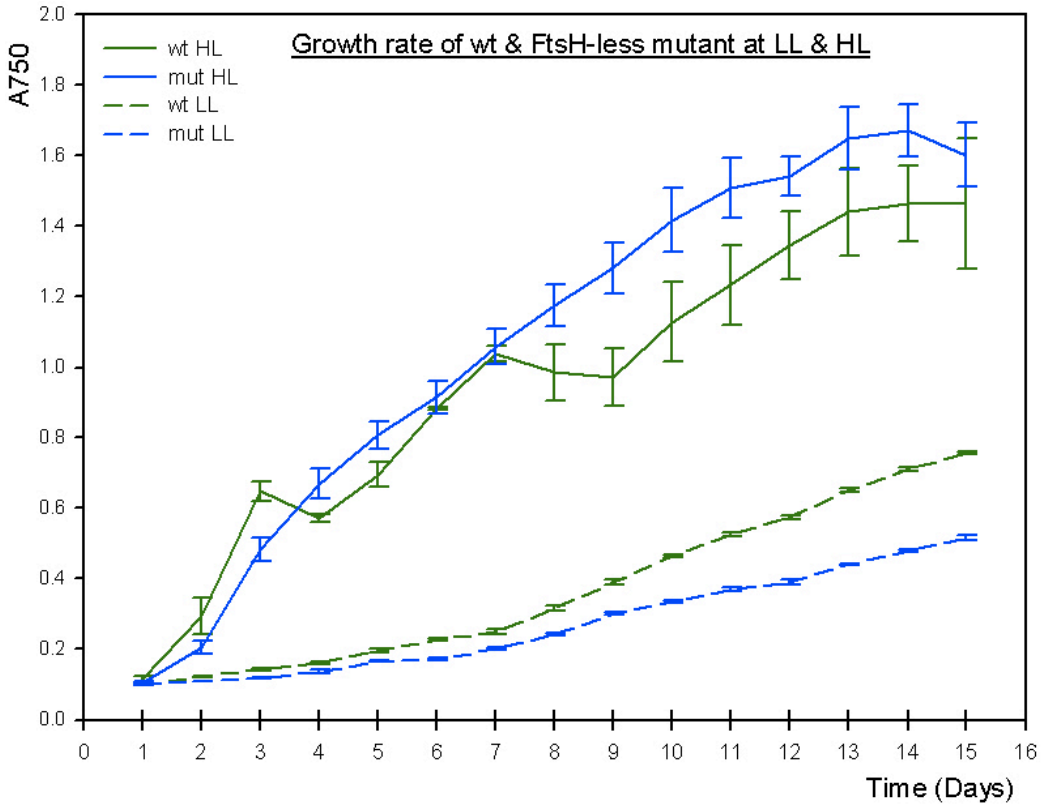

Figure IV.4. **Growth rate of wt and FtsH$^-$ mutant grown under Low (10 µmol. m^{-2}.s^{-1}) and High Light (160 µmol. m^{-2}.s^{-1}).** The mean values are the averages of at least five replicants, and the error bars again denote the standard error.

2.2.2. Pigmentation

As stated above the main reason for exposing the cells to irradiance as high as 160 µmol. m^{-2}.s^{-1}was to put the Repair Cycle of PS-II in our model cells into a serious test and record any changes in their phenotype.

The most striking difference appeared on the third day after the inoculation when the colour of the wt culture turned into a chlorotic yellow while that of the FtsH$^-$ mutant remained characteristically unchanged (Figure IV.5).

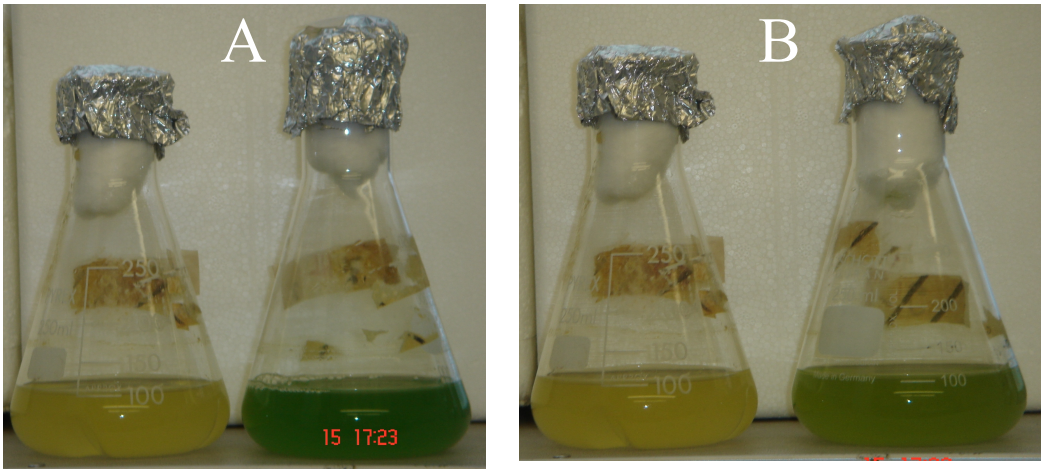

Figure IV.5. **Chlorosis during growth under High Light conditions**. From left to right: wt and FtsH$^-$ mutant. A). The picture is taken 72 hours after the inoculation. B). Ten days after the inoculation. The wt never recovered from the severe chlorosis appeared from the third day onwards, while in high contrast, the mutant remained green, chlorotic but nonetheless green until the last (fifteenth) day of the experiment.

The pigment content in each type of cells

The pigment composition of cells, wt & FtsH$^-$ mutant, grown under normal light conditions remained mostly unchanged although the former exhibited a slight tendency of less chlorophyll per cell during the phase of accelerated growth. In contrast, when the Repair Cycle was put to a test as the cells were exposed to high light, there was a profound change in pigmentation, most noticeable in wt which turned from green to yellow within three days (see Figure IV.5).

wt

Continuous illumination of cells with high light had a profound effect on cells' pigmentation; an intense chlorosis. The whole cell absorption spectra were made not so to confirm the evident changes in pigmentation but mostly to obtain a detailed picture of the process and quantify these changes.

The concentrations of chlorophyll and PC were calculated every day and values were plotted against time (see Figure IV.6). The graphs showed some interesting results.

While there is a marked increase in chlorophyll and phycocyanin accumulation in the culture during the first three days (for chlorophyll from 0.5648 μM on the first reading to 3.553 μM on the third, and for PC from 0.4133 μM to 1.3697 μM), the concentration of these pigments per cell for the same time remains

largely unchanged (Figure IV.6). This increase in the overall pigment concentration is believed to be due to the quick rise in cell number in the culture during this period.

However 48 hours after the inoculation, chlorophyll concentrations as per ml as per cell, drop sharply, exactly when the chlorosis of the wt took place and was recorded (Figure IV.5).

The chlorophyll concentration per cell falls by nearly 65% after 48 hours, from day 2 to 3, while the drop in PC content per cell, starts one day earlier than the chlorophyll's and within 48 hours declines by 80% (Figure IV.6)

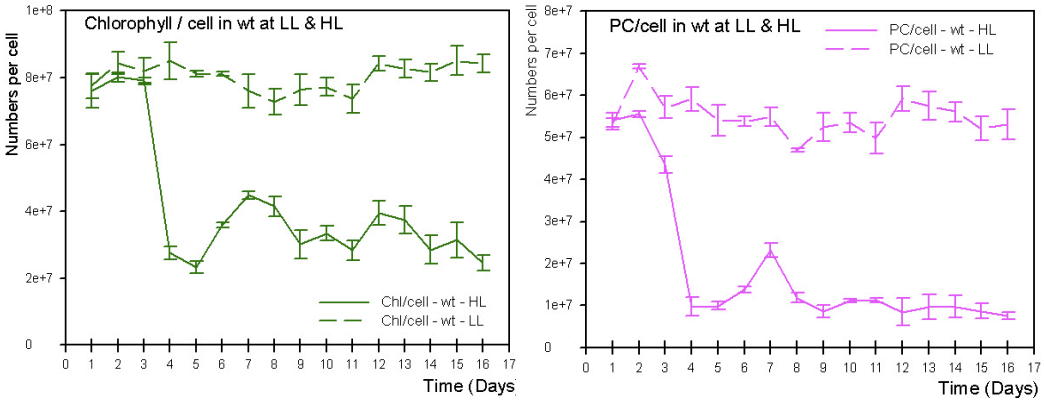

Figure IV.6. **Cellular concentrations of Chlorophyll and phycocyanin in wt type cells grown under normal and high light conditions**. The mean values are the average of at least three independent experiments and the depicted error bars represent the standard error.

FtsH- mutant

The pigment composition of cells lacking the FtsH protease was affected characteristically less than that of the wt cells. Despite the decline of pigment content per cell (Figure IV.7), the loss is not as steep as in wt, and for that reason the mutant cells preserved the green(ish) colour throughout the course of this experiment.

The chlorophyll concentration per cell remained largely unchanged for the first 72 hours after inoculation and declined only by 28% (from 6.144 *10^7 molecules per cell to 4.7* 10^7). The phycocyanin content per cell stayed mostly unchanged for the first 48 hours and the observed decline was less than 50% (5.6* 10^7 molecules per cell to 2.9* 10^7).

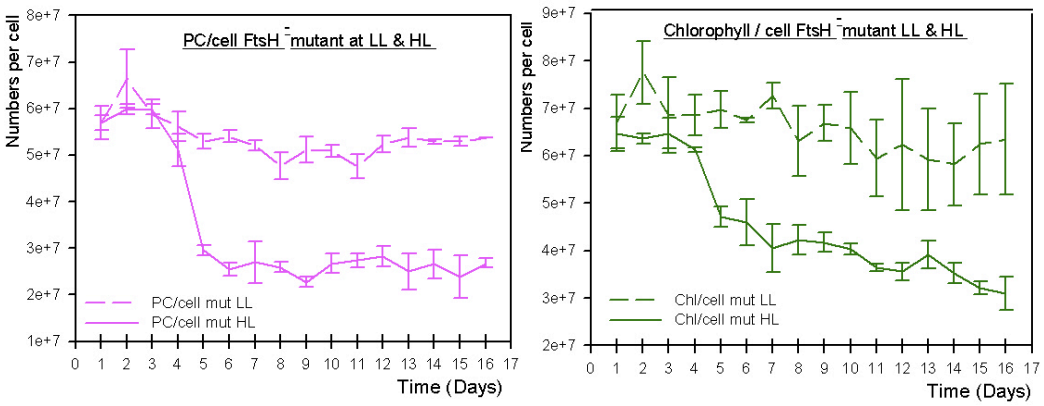

Figure IV.7 **Cellular concentrations of Chlorophyll and phycocyanin in FtsH⁻ mutant type cells grown under normal and high light conditions.** Mean values are averages of three minimum replicants and the error bars represent the standard error.

2.2.3. Differences between the two types of cells

Assessment of the wt and FtsH⁻ mutant cells grown under High light conditions revealed some interesting and important differences in their phenotypes.

As far as the growth rate is concerned, the results unveiled a noteworthy difference; a marked decline in the growth pace for the wt after the third day, while that of FtsH⁻ mutant remain principally unchanged and the latter appeared to grow faster than its progenitor wt.

The most profound however difference between the wt and FtsH⁻ mutant appeared in their pigmentation with an intense chlorosis afflicting mainly the wt cells soon after exposing both cultures to intense white light. (Figure IV.5 and IV.8)

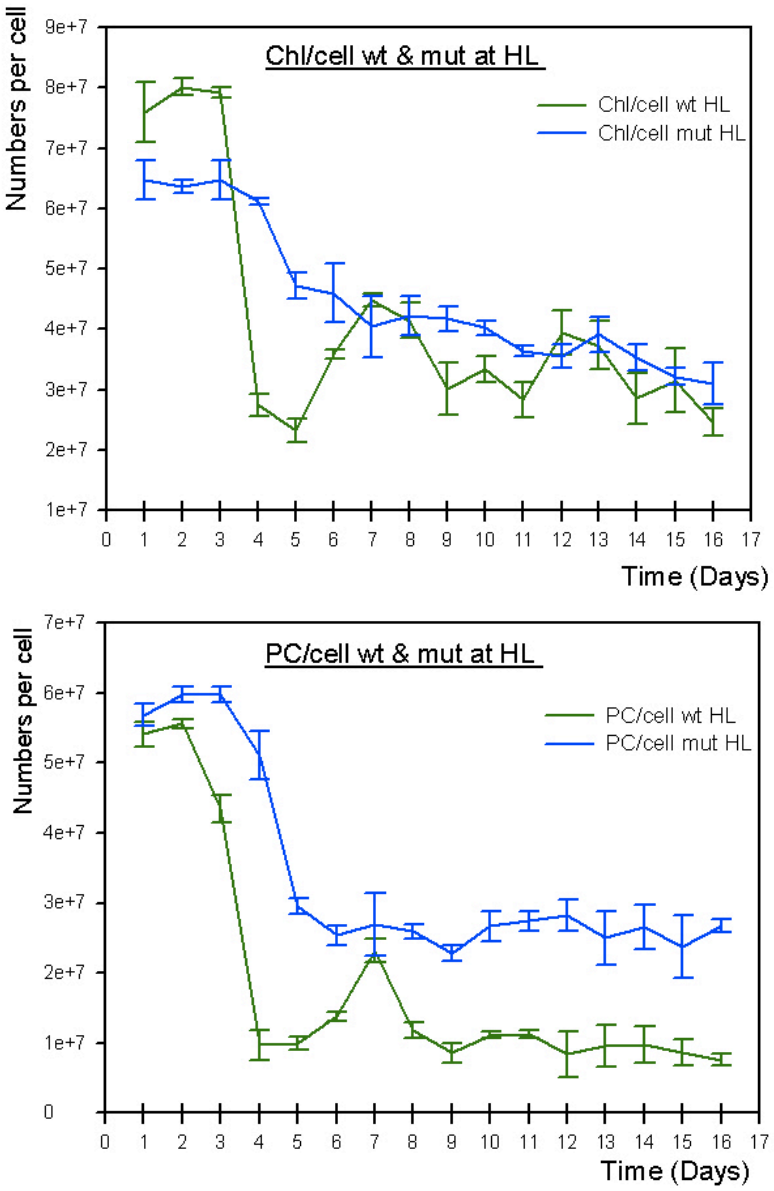

Figure IV.8. **Cellular content of Chlorophyll and phycocyanin in the wt and FtsH$^-$ less mutant, both grown under High Light.** Mean values are the result of at least five replicants. Standard error is represented by the error bars.

The chlorophyll concentration per cell in FtsH$^-$ mutant cell remained largely unchanged for 72 hours in comparison to 48 hours in mutant (Figure IV.8.). Yet in mutant it declined only by 28%, in comparison the impressive 65% in the wt cells.

The phycocyanin content per cell in FtsH$^-$ mutant also remained unchanged longer than in wt cell (Figure IV.6 and IV.7). The recorded fall was only 50% compared to the massive 85% in wt cells (Figure IV.8) .

3. Summary & Conclusions

Grown under normal incubatory light conditions, the mutant grows slower than the wt . The result certainly not predictable, is nonetheless expectable to some degree when considering the *modus operandi* and multifunctionality of FtsHases (Confalonieri & Dugout 95). Yet, given the multiplicity of FtsH proteases as in plants as in cyanobacteria, the possibility of functional overlapping cannot be excluded.

Both types of cells responded to high light with a marked increase of growth rate, Figure IV.4, compared to that under low intensity irradiance suggesting thus that the limiting factor for the relatively slow growth in the latter case was the intensity of light.

An interesting outcome of the high light experiment was the growth rate decline of the wt after the third day. Although difficult to explain sufficiently, given the limited amount of data, we propose an explanation for the observed phenomenon based exclusively on energetic cost for the repair and the cell division. As the rate of damage is proportional to the intensity of light, when the cells are being exposed to high irradiance for prolonged time the repair mechanism, operating in full capacity, is in high demand for energy. Wild type cells thereby spend a considerably higher amount of energy for the repair of the intensely photoinactivated photosystem-II, contrary to mutant cells where this function is severely depleted. In other words, the limited availability of free energy in wt may account for the observed decline of its growth pace.

The other intriguing phenomenon observed during growth under high light is the intense chlorosis affecting practically only the wt. In high contrast, the mutant cells remained green, chlorotic green, but nonetheless green. As surprising as it may be, this result is certainly explicable when considering the conditions of the experiment, the functional roles of FtsHases along with some known facts about the reaction of photosynthetic apparatus to different light intensities.

The content of a pigment that absorbs strongly under particular conditions appears to be contingent upon the intensity of light. Thus, while bright light appears to reduce the amount of pigment that strongly absorbs it, low irradiance on the other hand has the opposite effect on pigment's concentration (Ghosh & Govindjee *1966)*. Yet, the cellular chlorophyll content during PS-II repair is in

inverse relationship to the rate of D1 synthesis (Nixon *et al.,* 2010). Reflecting the significance of an FtsH ortholog in the repair mechanism of PS-II (Bailey *et al.*, 2002; Kato *et al.,* 2009; Silva *et al.,* 2003; Komenda *et al.,* 2006) with the above mentioned facts, then the severe chlorosis of the wt under specific light conditions is certainly a really positive indication of a functional repair mechanism which nonetheless can not keep pace with the rate of degradation. The severe chlorophyll degradation in wt is concomitant of the extensive damage to D1 subunit and of a Repair mechanism, that despite operating at its full potential, is insufficient to repair the damaged units under the specific conditions. In high contrast, the $FtsH^-$ mutant, which due to its depleted capacity for repair of inactivated units has an increased number of non-functional Photosystem-II (Chapter V) and therefore higher level of cellular chlorophyll.

CHAPTER V

THE FtsH PROTEASE AND ITS ROLE IN THE REPAIR CYCLE OF PS-II IN *SYNECHOCOCCUS* 7942

Chapter V

The FtsH protease and its role in the repair Cycle of PS-II in *Synechococcus* 7942

1. Introduction

Light, the ultimate driving force of photosynthesis, the source of almost all metabolic energy, can also be harmful for the photosynthetic apparatus. The phenomenon, known as photoinhibition, occurs whenever the normal electron flow is interrupted, namely when the excitation exceeds the capacity of electron flow.

To compensate for the light-induced loss, photosynthetic organisms have evolved a mechanism to repair the damaged site. In these terms, photoinhibition, or more precisely net photoinhibition occurs only when the photo-induced inactivation exceeds the capacity of the repair cycle at given conditions and the decline in photosynthetic performance can be observed and recorded.

Photoinhibition is a very complex phenomenon as along with the light's intensity and spectral composition, that alone are highly variable parameters, it also depends on time, abiotic stress conditions, and on the metabolic demands of the organism at a given moment (Huner *et al.,* 1998).

It becomes apparent that not only there is no clear cut line between efficient light harvesting and potential light-induced damage but yet the concept of "excess" light or excitation pressure does not correspond to any constant value.

In contrast to the intense debate about the causes of PS-II damage, there seems to be little doubt that the D1 core protein is the main site of photo-induced damage, and that members of FtsH proteases play a crucial role in the early stages of the repair cycle (Bailey *et al.,* 2002; Silva *et al .,*2003; Komenda *et al.,* 2006; *Kato et al.,* 2009; for review Nixon *et al.,* 2010).

Creation of an FtsH less mutant in a cyanobacterial species, that derives from the polyphyletic genus of *Synechococcus* (Robertson *et al.,* 2001), as our model organism, aimed at investigating the role of these proteases in the repair cycle and to propose (if this proved to be the case) a conserved role for these proteases in the repair mechanism among oxygen evolving photosynthetic bacteria. Yet more, given the size, shape and most importantly the spatial organisation of TM in *Synechoccocus* 7942, to explore

further the *in vivo* dynamics within the membranes and any possible involvement of FtsHases.

Despite the relative ease of creating mutants these days, especially bacterial ones, the interpretation of mutant data, most often than not, consists a complicated task with legions of factors to be taken into consideration before reaching a conclusion. Even a seemingly uncomplicated case cannot always be unambiguously interpreted, e.g. an unimpaired Repair Cycle in a FtsH null cyanobacterial mutant would obviously imply that the protease does not constitute an essential part of the repair mechanism. When however we take into account that, FtsHases have been shown to be involved it the repair mechanism, that all oxygenic phototrophs contain several of these proteases, and yet that hetero-oligomerization of FtsHases is a prerequisite for their function, then the foregoing fact might have other possible interpretations. An unaffected Repair Mechanism in an FtsH less mutant could not, and should not, automatically exclude the possibility that this particular protease is not involved in the repair cycle, when the organism lives in its natural environment, where the concept of natural, (for probably all living organisms, apart from us, *Homo sapiens*), is translated into a continuously changing and challenging environment.

2. Assessing the role of FtsH using Fluorescence Spectroscopy

Caused by emission of light from pigment molecules during transitions from excited to the ground state, fluorescence spectroscopy is a very useful method to study photosynthesis. The ability of the technique to be used in vivo, as well as *in vitro* systems, along with the fact that it can be measured in relationship to a number of parameters such as: temperature, presence of chemical reagents, pretreatment, time of illumination and others, made the method almost indispensable and analysis of the data can provide useful and reliable information about the kinetics in the electron transport chain, the efficiency of the photochemistry in reaction centres, the energy transfer and many others.

The technique was used regularly throughout this project. For consistency, samples of whole cells were adjusted to chlorophyll concentrations of 5 μg ml^{-1} in BG11 medium and were dark adapted for 5 minutes. For chlorophyll-*a* and PB excitation, wavelengths of 435nm and 600 nm were used respectively.

2.1. Emission spectra at 77K.

The *in vivo* Chl-*a* fluorescence emission at 77K from cyanobacteria as well as from red algae and vascular plants, are generally composed of three major bands, namely F_{685}; F_{695} and $F_{710\text{-}735}$ (Murata *et al.,* 1966; Govindjee & Yang 1966; Boardman *et al.,* 1966). The origin of each of these bands was assigned according to the action spectra obtained from purified PS-I and PS-II complexes (Murata *et al.,* 1966; Satosh 1980; Nilson *et al.,* 1992; Shen & Vermaas 1994). The first two bands, F_{685} and F_{695}, are from PS-II antenna pigments while the longer band $F_{710\text{-}735}$ is from PS-I antenna pigments (Murata *et al.,* 1966; Govindjee & Yang 1966; Boardman *et al.,* 1966). The latter is remarkably variable among different species in contrast to those from PS-II that are fairly constant. Although the complete assignment has not been fully established, most of F_{685} and F_{695} belong to Chl-*a* in core PS-II complexes, whereas $F_{710\text{-}735}$ to Chl-*a* in PS-I (Gasanov *et al.,* 1979, Rijgesberg *et al.,* 1979). Furthermore the F_{685} band is believed to originate from CP43 Chl-*a* and the F_{695} from CP47 Chl-*a* (Nakatani *et al.,* 1984)

Selective excitation of PC (phycocyanin) at 570-620nm, yields emission spectra with three peaks: the first peak at approximately 650nm is fluorescence from phycocyanin, and allophycocyanin; the following prominent peak (~680nm) is from PS-II and the PBS terminal emitters; the third one is from PS-I

2.1.1. Stoichiometry of PS-I relative to PS-II as recorded with 77K Fluorescence emission

Since the 77K $PSI/PSII$ fluorescence ratio changes during state transitions in cyanobacteria (Allen *et al.,* 1989; Bruce *et al.,* 1989; Vernotte *et al.,* 1990; Salehin & Bruce 1992) all samples in the current course project were dark adapted before freezing, to ensure that changes in fluorescence ratio between the two type of cells originated mainly from changes in the stoichiometry of photosystems I & II.

77K fluorescence emission spectra with excitation wavelength 435 nm (Figure V.1), for wt and $FtsH^-$ mutant show characteristically higher F_{685} and F_{695} bands in the mutant compared to those in wt. Yet, the ratio of relative fluorescence yield, F_{PSI} / F_{PSII}, has been significantly reduced, from 1.9 (±0.13) in wt to 1.03 (±0.17) in the mutant. The resulted decline in F_{PSI} / F_{PSII} ratio appears to be due to an increased fluorescence yield from PS-II and reduced from PS-I at the same time (Figure V.1).

The PSI/PSII ratio in cyanobacteria is a long term adaptation to changes in environmental conditions that along with CO_2 concentration, also depends on light intensity (Murakami & Fujita 1991) and the spectral fluctuations (Myers *et al.,* 1980; Fujita 1985). The stoichiometry of PSI/PSII may be seen as a regulatory mechanism maintaining efficient electron flow from PS-II to PS-I, that under low or moderate light growth conditions usually varies from 2 to 5 (Fujita *et al* 1994), whereas at saturating light intensities is around one (Murakami & Fujita 1991).

The higher PS-II fluorescence yield manifested by mutant, compared to that of wt, may be attributable to a slower D1 turnover rate because of the lack of FtsH protease (for review see Nixon *et al.,* 2010) and thus higher overall PS-II content in the cell i.e., sum of functional and non-functional PS-II reaction centres.

The 77K Chl-*a* fluorescence emission also shows a small but well noticeable blue-shift in the F_{PSI} fluorescence maximum, an average of 3nm (±0.5nm), in $FtsH^-$ mutant that correlates well with previous findings in *Synechocystis* 6803 (Mann *et al.,* 2000). Since changes in PSI/PSII stoichiometry, present in $FtsH^-$ mutant (for details see below), have not been reported to lead to such shift in F_{PSI} maximum, then this shift might be due to structural and functional changes in PS-I complexes. Therefore, it is plausible that the 360FtsH homologue may play a chaperon-like role in the assembly of functional photosystem-I centres.

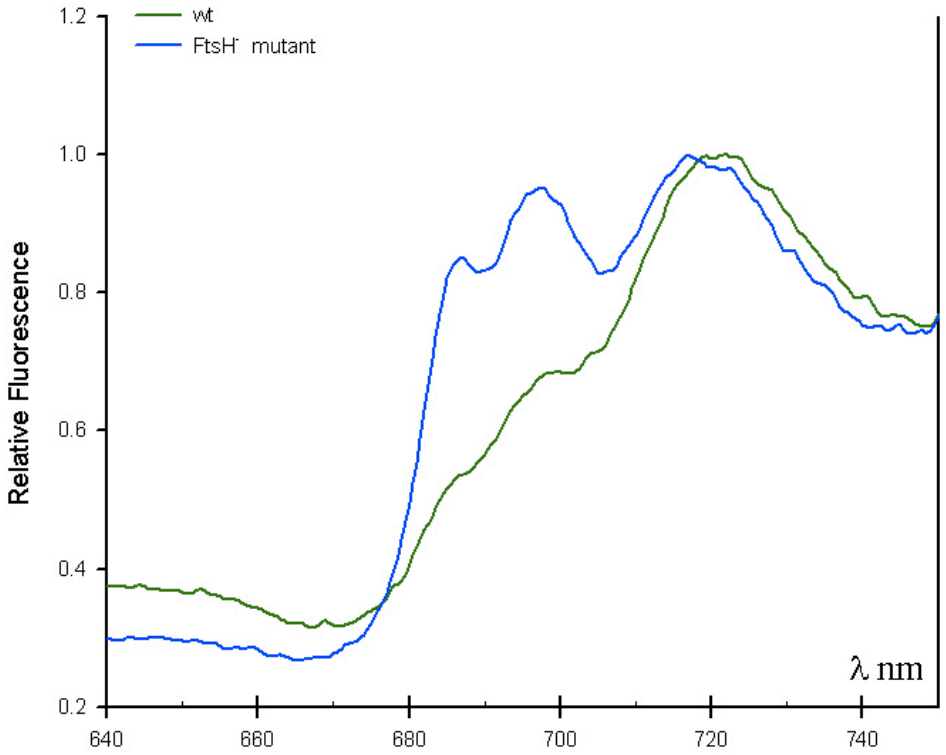

Figure V.1. **77K chlorophyll-*a* fluorescence emission spectra from wt and FtsH$^-$ mutant.** Data are normalised to the PS-I emission peak at 720nm. The illustrated here graph is representative of at least 6 replicants. In few cases the difference between PS-II and PS-I emissions in wt and FtsH$^-$ mutant was even larger, as PS-II bands in FtsH$^-$ mutant were higher than the PS-I (F_{725}) and with $F_{685} > F_{695}$. The spectra are normalised to PS-I peak because the absolute amplitudes of fluorescence emission spectra at these conditions are unreliable, despite the fact that the shapes are reproducible.

2.1.2. Light harvesting antenna and energy transfer

Photo-induced inactivation of PS-II causes a cascade of structural changes at both, acceptor and donor sides of the RC, e.g. loss of Mn, Ca and Cl atoms; no electron transport from Q_A to Q_B; phosphorylation of the damaged D1 subunit; detachment of LHC-II Chl-*a/b* in vascular plants (Baena-González *et al.,* 1999; Magnuson *et al.,* 1998; Haumann & Junge 1999) and others. Deletion of *fts*H gene in our model organism has evidentially affected the pigment composition of the cell, with 20 % less chlorophyll than in wt (Chapter IV). To examine the possibility if light-harvesting and energy transfer to reaction centres was affected in FtsH$^-$ mutant we also recorded the fluorescence spectra at 77K, with selective excitation of PB with 600nm wavelength, Figure V.2.

The presence of high fluorescent bands between 680 and 695nm, assigned to PS-II and terminal emitters in phycobilisomes, and also in the region ~720 nm assigned to PS-I, show an efficient energy transfer from PBS to the reaction centres, implying thus that

the light harvesting antennas remain correctly assembled with photosystems. Yet, given that the amount of PC present in both types of cells is nearly the same, the apparent higher fluorescence yield, region 680 - 695nm, in the FtsH$^-$ mutant, is another positive indication for a higher overall concentration of PS-II reaction centres. The latter is in all probability due to an increased proportion of non-functional PS-II complexes in FtsH$^-$ mutant, result of an impaired repair mechanism (Figure V.2).

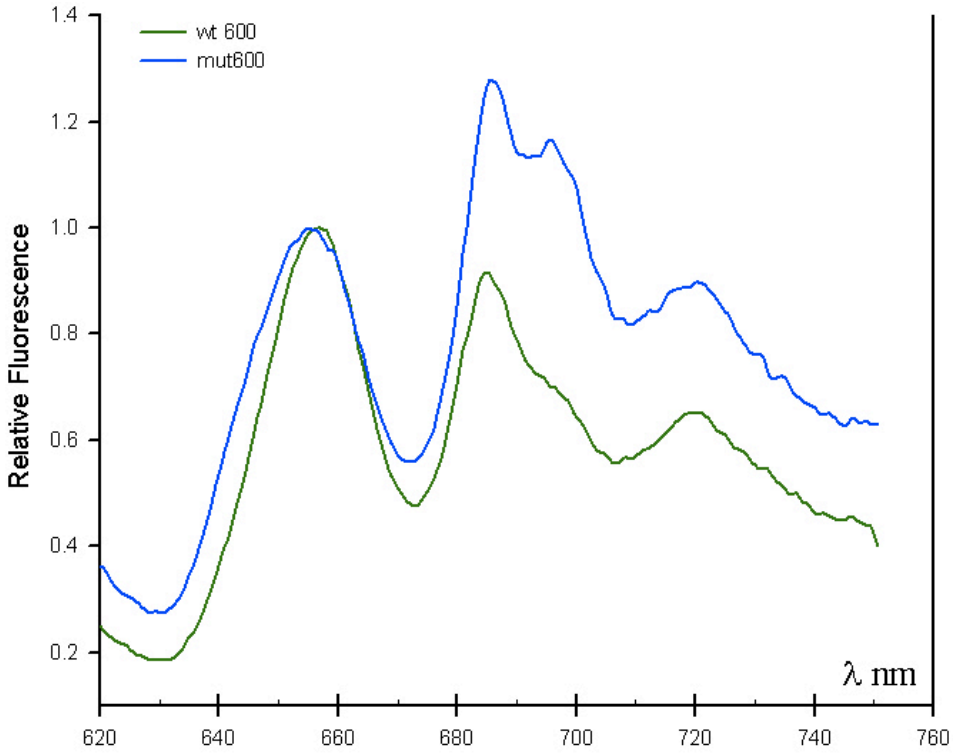

Figure V.2. **77K fluorescence emission spectra from wt and FtsH$^-$ mutant. Excitation wavelength 600nm.** Data are normalised to the emission peak of PC and APC at ~650nm. The results presented here are illustrative of at least five replicants. The spectra are normalised to PBS peak (~650nm) because the absolute amplitudes of fluorescence emission spectra at these conditions are unreliable.

2.2. Emission spectra at Room Temperature

At Room Temperature (RT) most of fluorescence emission comes from PS-II (Pfündel *et al.,* 1998; Gilmore *et al.,* 2000) as the lifetime of PS-I chlorophyll fluorescence is considerably shorter than that of PS-II (Krause & Weis 1991). Further, fluorescence from PS-I chlorophyll light harvesting antenna does not compete with the photochemistry of the photosystem (Gilmore *et al.,* 2000, Holzwarth AR 1991) and it is only the PS-II fluorescence that varies with changes in its photochemistry (Govindjee *et*

al., 1986). In cyanobacteria however there is a small but nonetheless non-negligible amount of fluorescence coming from PS-I as well, due to higher PSI/PSII ratio (Fujita *et al*. 1994) and the fact that PS-I have larger chlorophyll light-harvesting antenna (Mullineaux and Holzwarth 1993; Fromme *et al.,* 2001; Byrdin *et al.,* 2002).

Fluorescence emission at room temperature produces two main bands, one at 650nm and the other at ~ 685nm. The first peak at 650nm originates from PBS, PC and APC in particular, whereas the other at ~685nm comes form chl-*a* in PS-II and from long-wavelength pigments in PBS core (Sidler 1994).

The fluorescence emission at RT from wt and FtsH$^-$ mutant, with chl-*a* excitation at 435nm in Figure V.3., shows a higher peak at ~685nm in the FtsH$^-$ mutant. Yet another indication for a higher overall concentration of PS-II complexes. It correlates with the fluorescence emission data obtained at 77K; the previous findings (Bailey *et al.,* 2002; Silva *et al.,* 2003) and with the proposed role for the FtsH proteases in the repair cycle of PS-II (review Nixon *et al.,* 2010).

However, as in all ambient temperature observations, the emission peak at ~650nm originating from PBS (Figure V.3 A&B), was regularly higher in the wt, despite the decreased PS-II fluorescent yield, question was raised seeking elucidation. Why wt cells, while consistently exhibiting reduced PS-II fluorescence compared to that of FtsH$^-$ mutant, either at 77K or at RT, appear now with an increased phycobilisome fluorescence. Is this due to a larger size of PBS or / and to greater number of PBS in the wt and why?

To explain this particular fact we suggest that the phycobilisomes may be attached mostly to functional photosystems and therefore as the wt is legitimately expected to have a greater number of functional RC, it will for that reason contain a greater number of light harvesting antennas. Larger size phycobilisomes in wt is less likely given that both strains contain the same amount of PC in their cells (chapter IV).

The emission spectra with excitation at 600nm, Figure V.3.B, showing an efficient energy transfer from light harvesting to PS-II, are in support of this hypothesis as well. If phycobilisomes remain attached to photo-inactivated photosystems-II, then the band at ~690 nm, Figure V.3B, originating mainly from PS-II, should be expected to be higher in mutant than in wt as in all observations at 77K and at RT with chl-*a* excitation. Lower emissions peaks however at ~650nm and ~690nm in FtsH$^-$ mutant correlate well

with the proposed hypothesis of PBS being associated principally with functional photosystems-II, and thereby of fewer PBS in mutant cells.

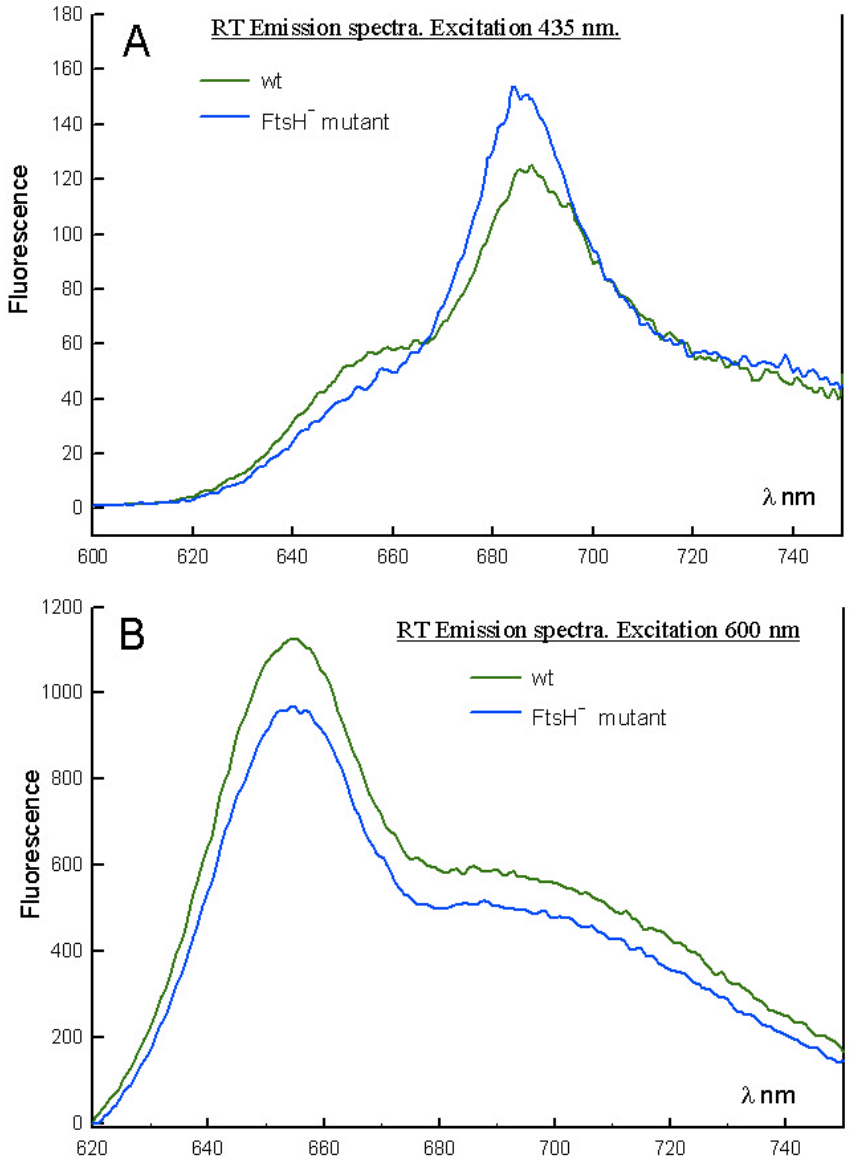

Figure V.3. **Room Temperature Emission Spectra with excitation at 435nm, for wt and FtsH$^-$ mutant.** Background fluorescence is subtracted. Graphs are not normalised. Chlorophyll concentration for both wt and FtsH- mutant were adjusted to to 5 µg ml^{-1} in BG11 medium and samples were dark adapted for 5 min prior to excitation. A) Emission spectra at RT with excitation wavelength at 435 nm. B) Excitation wavelength at 600 nm.

3. Content of Photosystem I & II

3.1. Concentration of Photosystem-I

As seen in low temperature emission spectra, Figure V.1, disruption of 360FtsH metaloprotease in our model organism *Synechococcus* 7942, caused a profound

reduction in fluorescence emission from PS-I relative to PS-II, suggesting thus a decrease in the abundance of photosystem-I. Using flash spectroscopy the cellular contents of PS-I in wild type and mutant were measured (Table V.1). The results substantiate the foregoing proposal of lower PS-I content in mutant cells that was found to be 80% of that in wild type (Figure V.4. & Table V.1). Yet, this data from flash spectroscopy are consistent with the previous findings in *Synechosystis* 6803 where using absorption difference spectra, the amount of PS-I in FtsHslr0228^{-} strain was also found to be significantly reduced (Mann *et al.,* 2000), leading thus to the suggestion of involvement of FtsH protease in the assembly of functional PS-I centres.

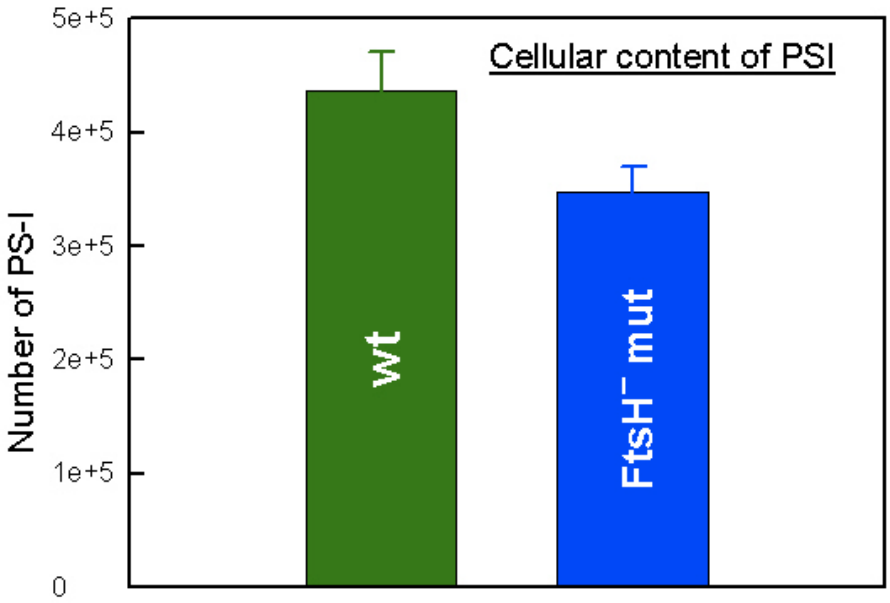

Figure V.4. **Photosystem-I content in wt and FtsH^{-} mutant. cells.** The error bars in the vertical charts represent the standard deviation.

Although the cellular content of chlorophyll in mutant cells is markedly reduced, 20% less, the number of chlorophyll molecules however per P_{700} in FtsH^{-} mutant, in comparison to wt, is increased, Table V.1.

3.2. Concentration of Photosystem-II

Along with plastoquinone, PS-II Q_B-binding pocket can bind the herbicide atrazine in molar ratio of one to one. This property of Q_B, is widely used to measure the concentration of photosystem-II by quantifying the ^{14}C- labeled Atrazine bound to Q_B site on D1 subunit (Wettern 1986; Stemler & Murphy 1984; Chow *et al.,* 1990).

Photosystem-II was assayed with the radioactively labeled herbicide, and the end result of this investigation showed a significantly reduced amount of these reaction centres in FtsH$^-$ mutant compared to that in the wild type (Figure V.5; Table V.1). The outcome is challenging to all spectroscopic data, either obtained at low or at ambient temperature. All emission spectra have unequivocally shown a pronounced increase of PS-II fluorescence in mutant (Figures: V.1, V.2 & V.3), proposing therefore legitimately greater overall number of photosystems-II in these cells.

Notwithstanding the predictions, the mutant appears to contain a notably reduced number of photosystems-II in its cells (Figure V.5), nearly by 50%, compared to that in wild type. To construe the results we proposed that the herbicide cannot bind the photo-inactivated D1 subunits and thus, it is principally the number of functional PS-II centres that is reflected through the atrazine assay.

Since the binding site of atrazine is located on D1 protein, that undergoes a series of conformational changes as a result of photo-induced inactivation (Baena-González *et al.,* 1999; Magnuson *et al.,* 1998; Haumann & Junge 1999) it is plausible that the Q_B binding cavity has been altered to that extent that the herbicide can only bind to those in functional PS-II. Indeed, specific residues in D1 protein have been shown to be the binding site of the herbicide since replacement mutations of these specific amino acids resulted in herbicide resistance-conferring mutations (Erickson *et al*.,1984, Erickson *et al.,* 1985, Diner *et al.,* 1991, Lardans *et al.,* 1997). This high specificity between the herbicide and the Q_B-binding pocket implies sensitivity of the binding ability of the atrazine to the conformational state of D1 protein, i.e., any alterations in the structure of D1, as a result of photo-inactivation, may prevent the binding of the herbicide. Amongst a series of such changes is the blockage of electron transfer from Q_A to Q_B, a self-implied in a way fact, that must occur to facilitate the replacement of the damaged D1 protein with a new one.

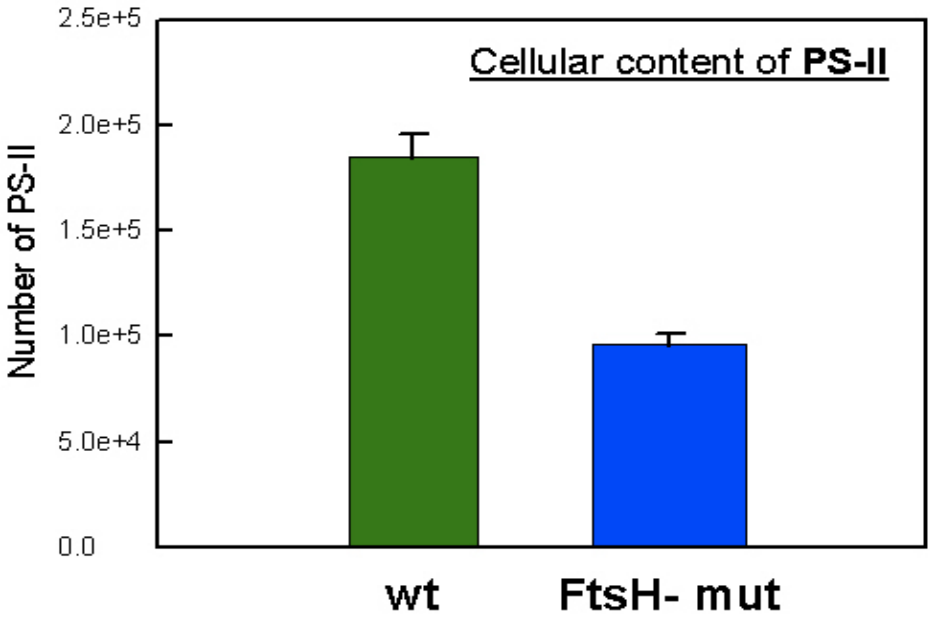

Figure V.5. **Content of PS-II centres in wild type and FtsH$^-$ mutant cells.** The error bars represent the standard deviation.

The research has shown that not only the herbicide binding changes with illumination (Jursinic & Stemler 1983) but that at ambient temperature the high light induced photoinhibition causes parallel decrease of functional PS-II reaction centres, the variable fluorescence (Fv/Fm), and the Atrazine's binding sites in spinach chloroplasts *in vivo* (Chow *et al.,* 1989). Since the intensity of light correlates well, not only with the rate of photo-induced damage to D1 but with the atrazine binding sites as well, it appears that assaying PS-II with ^{14}C-labeled atrazine concerns mainly the functional reaction centres. In support of this idea are also the findings that the herbicide will bind neither to D1 fragments nor to the precursor pD1 of D1 protein (Wettern 1986). Most importantly however is the existence of a good correlation between this method of radioactively labeled Atrazine and an alternative one assaying *in-vivo* functional PS-II centres by flash-induced oxygen evolution (Lambers *et al.,* 2008).

Thereby we propose that the results of this method reflect principally the number of functional PS-II centres which in turn explains the observed discrepancy between the spectroscopic data and the ^{14}C-labeled atrazine assay of PS-II. Mutant cells have a significantly reduced amount of functional photosystem-II complexes compared to those in wild type. However, the overall number of these reaction centres in FtsH$^-$ mutant, functional and non-functional is largely increased (77K & RT spectroscopic data), which in all probability is due to an impeded repair mechanism. The results

therefore reinforce the proposed idea of 360FtsH being involved in the degradation of photo-inactivated D1 protein and in the repair mechanism of PS-II.

	wt	FtsH- mut
Photosystem I / cell	4.36 *10^5 ± 48000	3.48 *10^5 ± 37000
Chlorophyll molecules / PSI	176 ± 10	213 ± 11
Photosystem II / cell	1.84 *10^5 ± 1500	9.5 *10^4 ± 5200
PS-I / PS-II	2.3	3.6

Table V.1. **Photosystem I and Photosystem II content in wild-type and FtsH$^-$ mutant *Synechococcus* 7942 cells**

The results of PS-II quantification with radioactively labeled Atrazine, if not supported with the spectroscopic data could certainly propose yet another function for FtsH protease, viz., involvement in the assembly of functional PS-II centres along with its role in the repair mechanism. Nevertheless, it is due to this, at first sight controversy, between the data from fluorescence spectroscopy and the quantification of PS-II using ^{14}C Atrazine that favours the proposed role in the repair cycle and makes it less likely to play a chaperone-like role in the assembly of functional PS-II centres.

4. Oxygen evolution and the role of 360FtsH protease

Since the hypothesis of this project is the involvement of FtsH protease in the repair mechanism of PS-II, and yet, the photosynthetic performance of FtsH$^-$ mutant under normal conditions appeared to be satisfactory despite the number of changes in the stoichiometry and composition of its photosynthetic apparatus, we tested the operation of PS-II and the repair mechanism under more challenging conditions. For the purpose of this investigation, a large series of measurements of PS-II activity, assayed by the rate of oxygen evolution, were recorded as a function of time that the cells were exposed to photoinhibitory conditions with and without lincomycin.

The aforementioned antibiotic blocks *de novo* protein synthesis, and thereby prevents the repair of damaged D1 proteins (Silva *et al*., 2003). In other words, it disables completely the repair mechanism of PS-II. Thus, under photoinhibitory conditions, decrease in oxygen evolution in the presence of lincomycin is in a way, equivalent to

the rate of photo-induced inactivation of PS-II. Higher oxygen evolution rate in the absence of lincomycin, compared to that with the antibiotic, is demonstrative of an active repair mechanism.

The effect of lincomycin on the rate of oxygen evolution in wild type cells becomes apparent, Figure V.6.A., as in nearly 25 minutes, it declines to 50%, whereas in ~50 minutes no PS-II activity could be recored any longer. In high contrast, the influence of an active repair mechanism is discernible when the cells are photoinhibited without the antibiotic (Figure V.6.A). Despite the evident decrease of PS-II activity in the absence of lincomycin, it remains considerably higher throughout the duration of the photoinhibitory treatment compared to that with the antibiotic present. Thus for the same period of 25 min and with the repair mechanism intact, i.e. absence of lincomycin, the decline in the rate of oxygen evolution is mere 10%. The pronounced difference in oxygen evolution rates in two cases, signifies the existence of an active repair mechanism that even after 60 minutes of photoinhibition can sustain as low as 10% of the initial rate of oxygen evolution. It is noteworthy that in three instances when the duration of the photoinhibitory treatment, without lincomycin, was purposefully prolonged, the wild type cells appeared to be capable of sustaining that minimal rate, precisely 9.4% ± 1.07, for another 20 minutes.

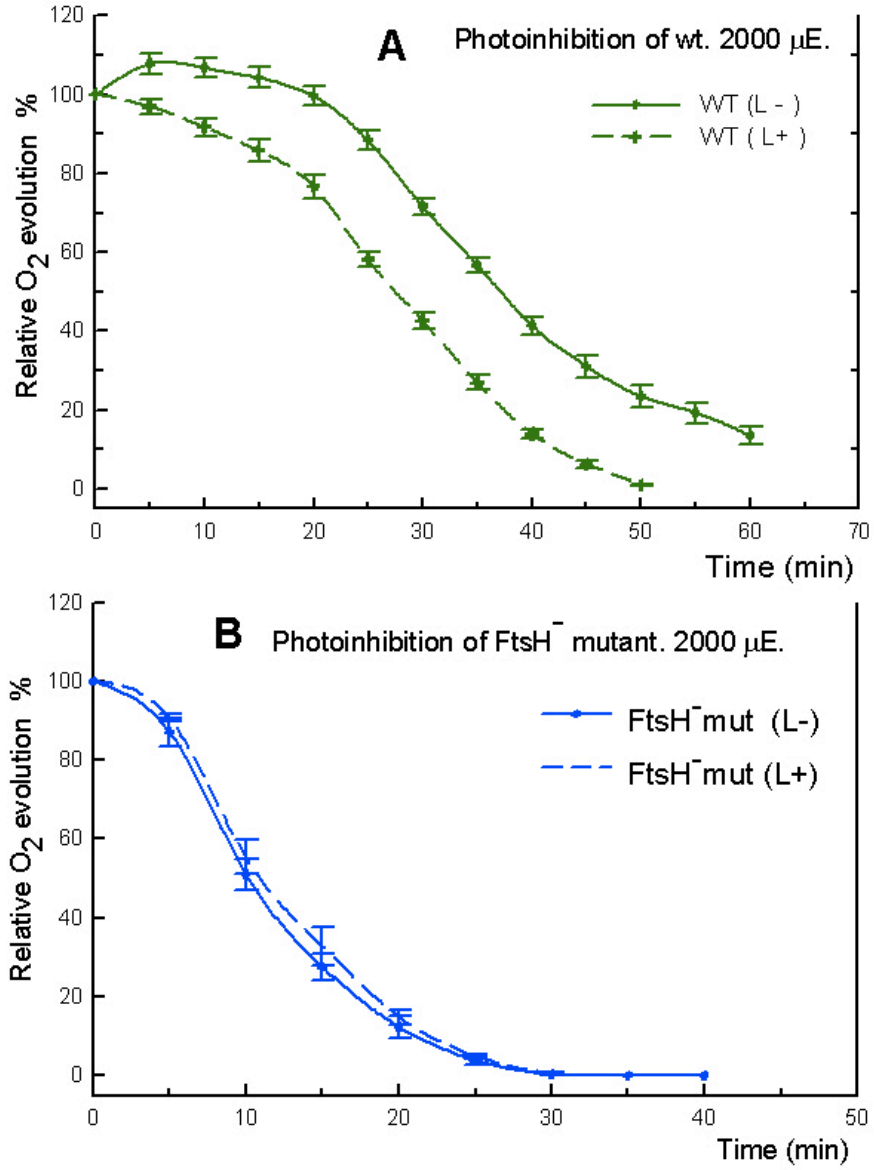

Figure V.6. **Relative Oxygen evolution of wt and FtsH- mutant under photoinhibitory conditions.** The oxygen evolution is relative to the rate prior to photoinhibiton. The graphs are the means of 15-20 replicates. The error bars in both graphs indicate the standard deviation. The temperature in oxy-electrode chamber were adjusted to 30 ^{o}C and the Light intensity to 2000 μmol. $m^{-2}.s^{-1}$. A) Relative oxygen evolution of wild type cells in the presence (solid green line) and absence (dashed green line) of lincomycin . B) Relative oxygen evolution of FtsH^{-} mutant in the presence (dashed blue line) and in the absence (solid blue line) of lincomycin.

Furthermore, the noticeable initial increase in the rate of oxygen evolution in the absence of lincomycin (not observed when antibiotic is added), might be ascribed to adaptation period that requires protein synthesis.

The profound effect of an active repair mechanism on PS-II activity, plainly evident in wild type cells when exposed to high light, is not observed in mutant despite the large number of the replicates of the experiment, Figure V.6.B. With and without the antibiotic the decline in oxygen evolution is identical in both cases. Even with an active protein synthesis (absence of lincomycin) and theoretically unrestricted repair mechanism, the rate of damage to PS-II under the photoinhibitory conditions, matches completely the one when the repair cycle is practically blocked due to lack of protein availability in the presence of lincomycin.

The loss of FtsH protease has undeniably impeded the ability of the cells to repair the damaged PS-II complexes, Figure V.6.B. This consists a strong indication that 360FtsH is involved in the repair mechanism of photo-damaged photosystems-II.

The presence and to one extent the significance of PS-II repair mechanism became apparent when the rates of relative oxygen evolution from cells with an active and disabled repair cycles were plotted against each other as a function of time, Figure V.6. A & B. Nevertheless, in these graphs, despite the obvious photoinhibition, the recovery of PS-II activity as a function of time, is somehow less obvious.

The area between the two graphs, namely between that with lincomycin present and the one when absent, Figure V.7., represents in fact the capacity of the repair mechanism, whereas the difference between the two rates of relative oxygen evolution, plotted against time gives the activity rate of recovery, Figure V.7.

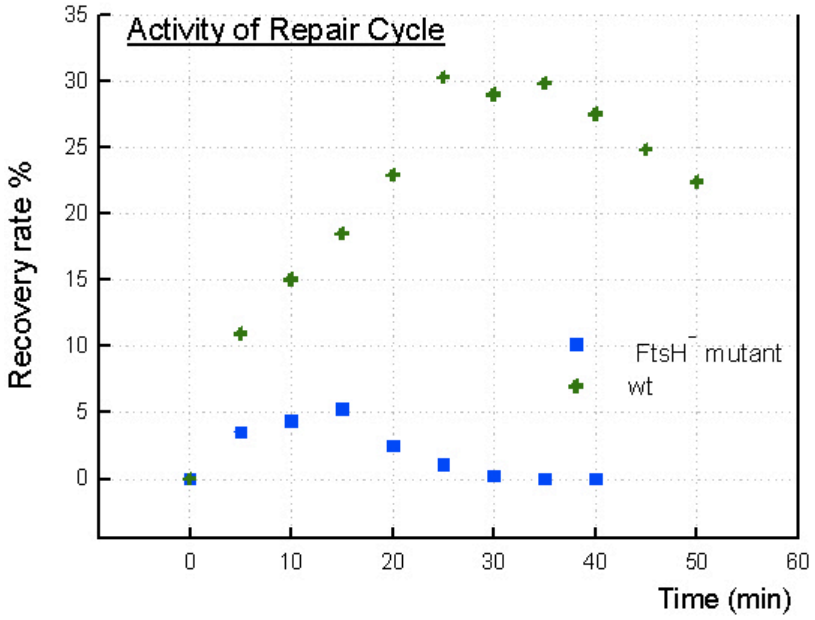

Figure V.7. **Activity rate of oxygen evolution recovery.** As O_2 evolution is a by-product of PS-II activity, then monitoring the former is though indirect, but nonetheless secure way to observe the repair mechanism. The graphs for each type of cells were constructed by subtracting the data of oxygen evolution with lincomycin added from that with lincomycin absent.

The results, Figure V.7, show that the recovery of lost PS-II activity in $FtsH^-$ mutant is practically nonexistent. In high contrast, in wild type the recovery of PS-II is not only evident but it also rapidly increases with the time, to reach its maximum after ~ 25 minutes when the repair mechanism recovers ~30% of lost PS-II activity. Combining the information from graphs in Figures V.6 and V.7, it can be inferred that as the number of PS-II being damaged during high light treatment increases, so does the number of repaired complexes. Although the damage is not paralleled with the repair in absolute numbers, it is noteworthy that the capacity of the repair cycle does reache its maximum not when the rate of relative oxygen evolution is higher than the initial one, Figure V. 6A, but actually when it is significantly lower, viz., after ~25 min, and sustained at that maximum level for nearly another 15 minutes, before it eventually started to decline.

In conclusion, the repair mechanism is a time dependent process that attains maximum efficiency when the overall PS-II activity has been already significantly reduced due to photo-induced damage. Yet, the efficiency of the repair mechanism, although can remain at the highest levels for a considerable length of time, eventually starts to decline, indicating thus that the repair mechanism itself is stress (high light) dependent process. Indeed, the repair mechanism has been shown to be contingent on stress

conditions (Takahasi & Murata 2008), a fact aggravating to one or another degree the rate of recovery of functional PS-II centres.

The ability of the cells to sustain a minimum yield of oxygen evolution for a prolonged period of time, i.e., ~10% of the initial rate even after 80 min of photoinhibitory treatment, indicates that the repair cycle, despite its reduced efficiency, remains active for considerably longer span of time.

To assay the impact of 360FtsH loss on the repair mechanisms in *Synechococcus* 7942, we compared the rates of oxygen evolution, in the presence and absence of lincomycin, between the wild type and the $FtsH^-$ mutant.

With protein synthesis intact, the observed difference in rates of oxygen evolution of wild type and the mutant simply confirmed the prediction, Figure V.8.A. The profound effect of 360FtsH presence in the wt strain, is plainly evident: markedly higher rates of oxygen evolution throughout the duration of the experiment; threefold longer time required to induce the same damage to PS-II activity; no complete damage to PS-II observed, even after 80 minutes of continuous high light stress. In high contrast, in $FtsH^-$ mutant, there is sharp and fast reduction of oxygen evolution, which after ~25 minutes is practically nonexistent. The large difference in rates of oxygen evolution between the two strains, is but another undeniable indication that the 360FtsH protease plays an essential role in the repair mechanism of photo-damaged PS-II.

The rate of photo-induced damage to Photosystem-II depends on the light dosage, intensity and time of illumination, (Anderson 2001). In the presence of lincomycin, that prevents the repair of damaged photosystem-II complexes by blocking protein synthesis, the rates of PS-II deactivation in wt and the $FtsH^-$ mutant are expected, theoretically at least, to be similar. Nevertheless, after a series of oxygen evolution measurements, performed under high light intensities and with the repair mechanism deactivated because of lincomycin, it appeared that the rates of PS-II photo-damage differ significantly between that in wt and the mutant (Figure V.8.B)

Unlike *slr*0228::Ω mutant in *Synechocystis* 6803, where the rates of loss of PS-II activity, under saturating light irradiance and in the presence of lincomycin were found to be similar in both wt and the mutant (Silva *et al.,* 2003), deletion of orthologous 360FtshH in *Synechococcus* 7942 had apparently a more dramatic impact, not only on the repair cycle but on the cell as a whole.

The insertional mutagenesis in our model organism has undoubtedly caused a cascade of structural and functional changes in the photosynthetic apparatus of the cell: slower growth rate; lower chlorophyll concentration; impaired repair mechanism; reduced number of functional PS-II; increased mobility of PS-II within TM; possible involvement in degradation of functional PS-II under nutrient stress (for details see below); reduced concentration of PS-I along with some structural & compositional changes. Despite the importance all these factors have individually or collectively for the performance of the photosynthetic, none of them can provide a rational explanation for the recorded difference in rates of oxygen evolution illustrated in Figure V.8.B. The massive reduction in the number of functional photosystems-II in the mutant, nearly half compared to the wild type, can however explain to certain degree the 50% reduction in time, required to bring about the same decline in the rates of O_2-evolution in both wt and mutant. Thus for instance, the time required to reduce the PS-II activity to 10% in mutant is ~20 minutes, whereas the same reduction in wild type is achieved after forty plus minutes (Figure V.8.B).

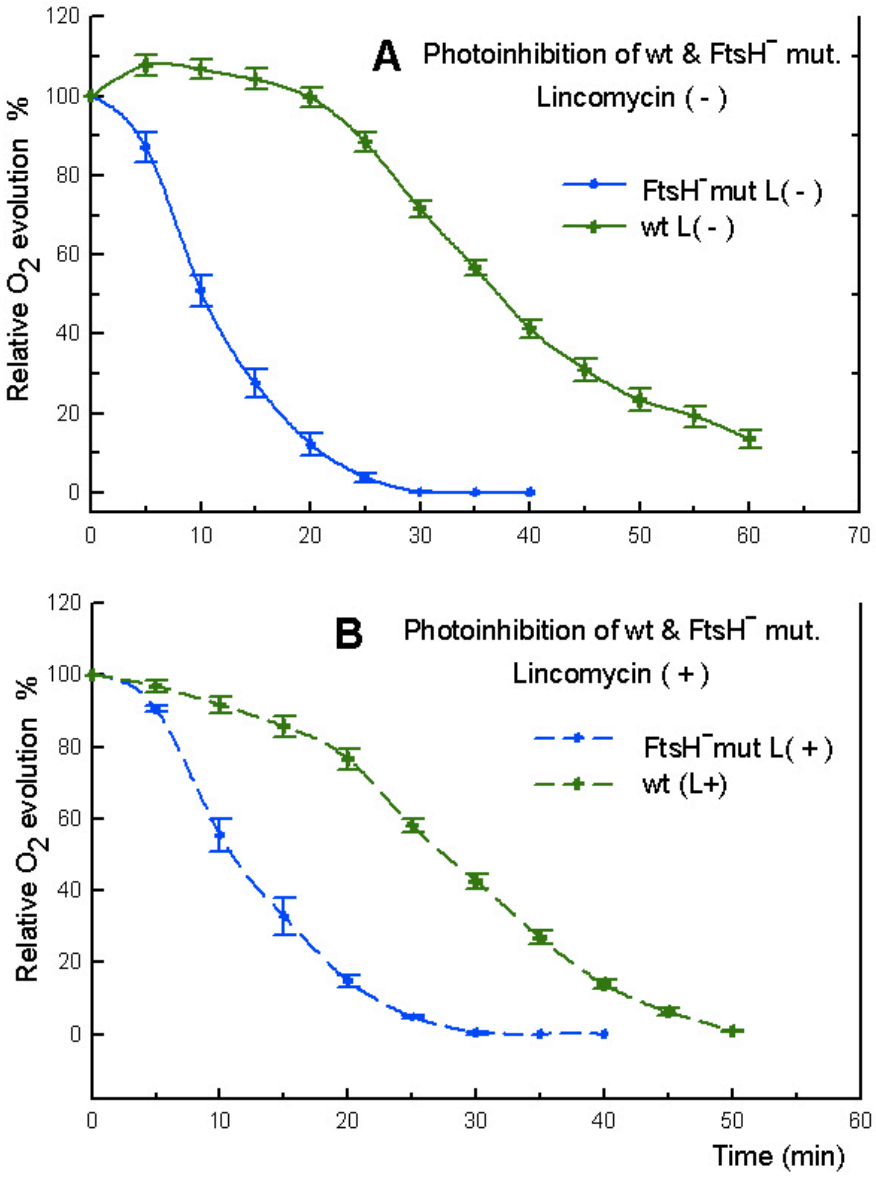

Figure V.8. **Comparison of relative oxygen evolution rates between the wt and FtsH-mutant.** The oxygen evolution is relative to the rate prior to photoinhibiton. The graphs are the means of 15-20 replicates and the error bars in both graphs indicate the standard deviations for each point. A) Comparison of the relative oxygen evolution rates in wt and FtsH- mutant in the absence of antibiotic lincomycin. B) Comparison of the two rates in the presence of lincomycin.

Another factor that may contribute to certain degree to this large difference might be the size of light harvesting antennas in each strain. Given that both wt and FtsH- mutant contain nearly the same amount of PC in the cells, yet the mutant is likely to contain a reduced amount of PBS, attached as proposed principally to functional Photosystems-II, the size of the antennas in the mutant therefore may be larger than in wt. Bigger size PBS, if this the case, can certainly contribute to faster deactivation of functional photosystems-II in the mutant. Nonetheless, the size of the antennas is unlikely to be the sole reason for the observed difference in oxygen evolution rates.

Taking into consideration the knowledge about FtsH proteases; multi-functionality, multi-gene family, pattern of proteolytical action in hetero-oligomeric ring-like structures; and combining it with the number of changes the deletion of this FtsH protease has caused in *Synechococcus* 7942, it is plausible that the overall fitness of the mutant has changed so, it is now more prone to photoinhibition.

5. State transitions

Life of photosynthetic organisms in their natural habitats is life under continuously fluctuating light and stress conditions. As photosystems I & II have distinct light absorption properties, but work in series to produce reducing power for assimilation of atmospheric carbon, then finding a fine balance between efficient light harvesting and potential photodamage, means finding a way of redistributing the absorbed excitation energy between PS-I and PS-II so the light limited photosystem receives more whereas the light saturated - receives less. State transitions are such short term physiological adaptations and as a phenomenon are ubiquitous amongst oxy-phototrophic organisms (William and Allen 1987; Allen 1992). In cyanobacteria, redirection of absorbed energy between two photosystems can be achieved by transient interaction of PBS with either photosystem (Mullineaux 1992; 1994; 1999). State-1 transition involves re-direction of absorbed energy from PS-I to PS-II and vice versa, state 2, from PS-II to PSI.

Given that in experimental environments it is possible to provide the spectral bands selective for each photosystem, 77K fluorescence emission is then an excellent means to record these transitions. For the purpose we plot two emission spectra graphs for each type of cells that prior to excitation have been adapted in dark to induce state-2 and in red light for state-1, Figure V.9.

If in State-1 the fluorescence emission from PS-II is greater than when the cells are adapted to State-2, this indicates that despite the preferential selection of PS-I with far red light, a higher proportion of this energy is transferred to PS-II and the cells are capable of performing state transitions (Murata 1969).

The State-1 transitions, i.e. energy transfer from PS-I to PS-II, reflected as rise in fluorescence from PS-II bands is clearly seen in wt as well as in FtsH$^-$ mutant cells in Figure V.9. From the graphs becomes evident, that lack of 360FtsH protease does not affect, in any apparent way at least, the ability to perform state transitions and thus to redistribute efficiently the excitation energy between the two photosystems.

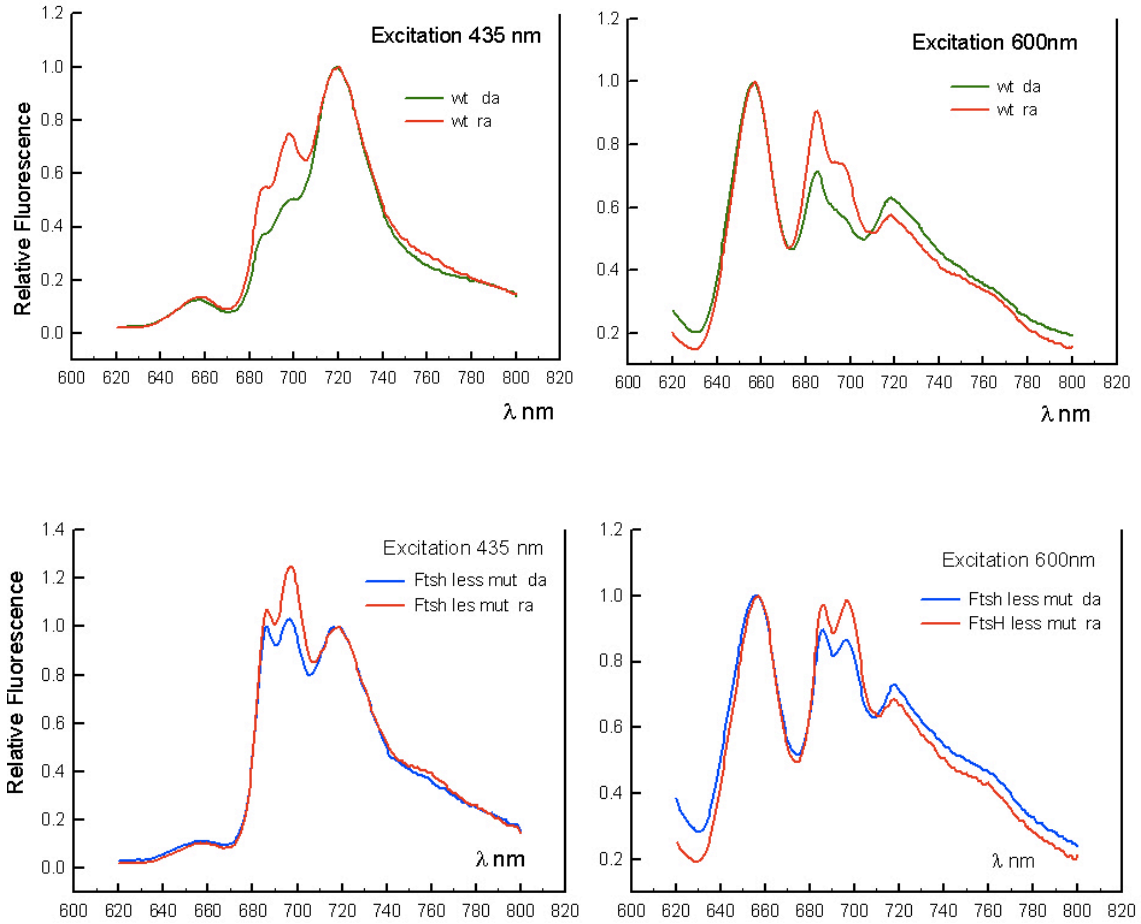

Figure V.9. **State transitions in cells grown under normal light conditons. 77K Fluorescence Emission Spectra**. Before freezing in liquid nitrogen and recording the fluorescence emission, both types of cells were adapted to state 1 & 2, i.e. left under red light and in the dark respectively for 5 minutes. Red lines for both types, wt and FtsH$^-$ less mutant, represent state-1, i.e., red adapted (ra) while the green and blue lines represent dark adapted (da) cell of wt and FtsH$^-$ mutant respectively. Fluorescence spectra were recorded with excitation at 435 and 600nm and then normalised to PS-I (~720nm) and PC (~650 nm) respectively.

Nevertheless the rise of fluorescence in PS-II peaks from dark to red adapted cells (State-1 transitions, Figure V.9), is similar in wt and FtsH$^-$ mutant. Given that there is a large proportion of non-functional PS-II in the mutant, but the rise in fluorescence during state transitions is very similar to that of the wt, these facts combined may consist an indication that the non-functional PS-II complexes do not participate in state transitions, either because PBS somehow recognise the non-functional PS-II and therefore do interact only with functional complexes or because the non-functional and functional PS-II are not adjacent as the former have migrated to the repair zones or are being repaired.

To examine this possibility one step further we performed the state transitions experiment as above, but with a sole difference that both type of cells were cultivated under high light. As PS-II inactivation *in vivo* occurs at all light intensities, and depends on the number of photons absorbed (Anderson 2001), growth under high irradiance will inevitably increase the rate of D1 inactivation, i.e. proportion of photodamaged PS-II complexes. The wt cells of our model organism, when cultured under such conditions, exhibited intense chlorosis in hight contrast to the mutant cells that remained green throughout the duration of the experiment. This was a positive indication that under such irradiance the repair mechanism can not keep pace with the rate of photo-inactivation, hence the population of non-functional PS-II is expected to increase and the difference in fluorescence from PS-II bands during state transitions from dark to red adapted cells to be higher. Cells grown under high light not only did not exhibit this fluorescence increase in PS-II area, but the difference even got smaller, Figure V.10., especially in wt cells.

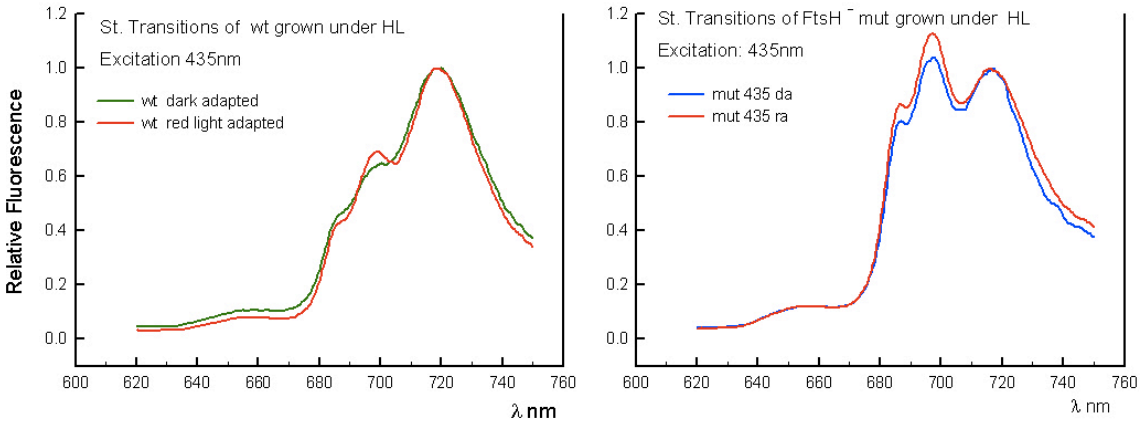

Figure V.10. State transitions in cells grown under high light. 77K Fluorescence Emission Spectra. Excitation wavelength 435nm. The same conditions and procedures were followed as before (see above, Figure V.9) with a single differences that this time both, wt and FtsH- mutant, were cultivated under high light conditions.

Although both type of cells are capable of redistributing the energy between the two photosystems, the little difference in PS-II fluorescence bands between state-2 and state-1, especially in the wt cells this time, is another positive indication that PBS are likely to redistribute the excess energy only to functional PS-II reaction centres. This is probably because PBS cannot engage even transiently with inactivated complexes or because the latter are moving to the repair zones or are being repaired (attachment of FtsH-complex on the stromal side). This proposal is consistent with the idea suggested

earlier on in this chapter that PBS appear to be principally attached to functional photosystems-II.

However, an alternative explanation to the above, may be the possibility that state transitions are required and performed only under very low light irradiance to maximise the efficiency of light harvesting as they are not part of the protective mechanism against photoinhibition (Emlyn-Jones et al 1999; Mullineaux & Emlyn-Jones 2004).

6. Response of photosynthetic apparatus to N-starvation

Inhibition of photosynthetic activity is a time dependent process that is contingent upon light and other abiotic stress conditions and the metabolic demands of the organism at any given moment. Yet, along with the inhibition of photosynthetic activity, stress conditions have been shown to affect the repair mechanism of PS-II (Takahashi & Murata 2008), hindering even further the process of recovery.

Abiotic stress conditions as nutrient deprivation, have been demonstrated to lead cyanobacteria to modify their photosynthetic apparatus, not only by degrading their PBS content (Yamanaka, & Glazer. 1980; Collier & Grossman 1992) as a general aspect of nutrient-limited growth (Reithman *et al* 1988), but also result in loss of PS-II activity and formation of non-functional PS-II complexes (Grossman *et al.,* 2000; Steglich *et al.,* 2001)

We raise the question whether 360FtsH protease plays a more general role in the quality control mechanism of TM under stress conditions as for instance nitrogen starvation rather than that caused by photo-induced inactivation of D1. To investigate whether the deprivation of cells from an essential element as nitrogen, would affect photosystem-II complexes (apart from the expected impact on PBS content), absorption spectra, 77K & RT fluorescence emissions were recorded and the cellular concentration of PS-II using radioactive Atrazine were also calculated.

For the purpose, both, wt and FtsH^{-} cells, were grown as before, but in medium lacking any Nitrogen ingredient. Light was adjusted to 10 μmol. $m^{-2}.s^{-1}$, so if any breakdown of photosynthetic apparatus, it will be mainly due to nutrient deprivation rather than to light-induced damage.

6.1. Pigment composition during Nitrogen Deprivation

Environmental conditions such as light intensity and spectral composition, temperature and nutrient availability are all, individually as well as collectively, essential parameters

that determine the performance of photosynthetic machinery, the stoichiometry of its complexes and the composition, both qualitative and quantitative of pigments and the proteins they are associated with (Fujita *et al.,* 1994; Grossman *et al*., 1995)

The phenomenon of chlorosis appears to be a general response to limitations for any number of different nutrients and is due to changes in pigments composition and content. Lack of nitrogen or limited availability of this essential element provokes a series of diverse, structural and functional changes, but as far as the photosynthetic apparatus of cyanobacteria is concerned it undeniably induces intense loss of phycobilisomes (Lau *et al.,* 1977*,* Yamanaka & Glazer 1980). As PBS may represent up to 50% of cyanobacterial cellular protein (Allen 1984; Bryant 1986), in time of nutrient deficiency they can be used as intracellular store for nitrogen (Allen & Smith 1969; Boussiba & Richmond 1980). However the way and the extent to which an organism will utilise its PB content as source of nitrogen under insubstantial availability of the element in the medium, can be a species specific characteristic (Steglich *et al.,* 2001).

Although degradation and loss of PBS are often used interchangeably as synonyms, often these two are not necessarily identical processes (Collier & Grossman 1992) since loss may be due to degradation and / or repressed synthesis. PBS degradation however, that is itself a multistep process (degradation of the terminal PC hexamers, whole rods, and the breakdown of the core), may allow the recycling of amino acids into proteins that are more important for the cell functioning and survival under these conditions.

In the current investigation, the pigment content (PC & chlorophyll), have been quantified as per cell as per volume culture (μM). For the purpose, room temperature whole cell absorption spectra were performed at equal time intervals. Distinguishing the two types of concentrations, viz. cellular and per volume of culture, was made on the basis that it can facilitate the interpretation of the data.

In contrast to loss of PBS commonly observed during N deprivation, the chlorophyll content under the same conditions is an issue under debate. In our investigation, no net chlorophyll degradation (per volume of culture, μM) has been observed in either type of cells, Figure V.11. The wt cells exhibited even a slight increase in the overall content of chlorophyll (μM), whereas in the FtsH$^-$ mutant it remained largely unchanged throughout the experiment, Figure V.11.

Unlike the overall concentration of chlorophyll, the cellular content of this pigment, in both wt and FtsH$^-$ cells, exhibited, in nearly the same way, the tendency to decrease,

Figure V.11. Given that no net chlorophyll degradation was observed, this decline in cellular chlorophyll is most likely attributable to cell division.

In high contrast to chlorophyll, the nitrogen-limited growth had an immediate and profound impact on PC content (Figure V.11. A & C) correlating well with previous findings (Yamanaka & Glazer 1980). Decline of the cellular PC exhibited a remarkable similarity in both wt and FtsH$^-$ mutant. Any differences however in the concentration of PC per volume of culture are almost certainly attributable to differences in cell numbers. As a result, the PC/Chl ratio, either as a measure per cell or per culture volume declined rapidly, by 80% within 96 hours, in almost identical pattern in both wt and FtsH$^-$ mutant (Figure V.11. B & D).

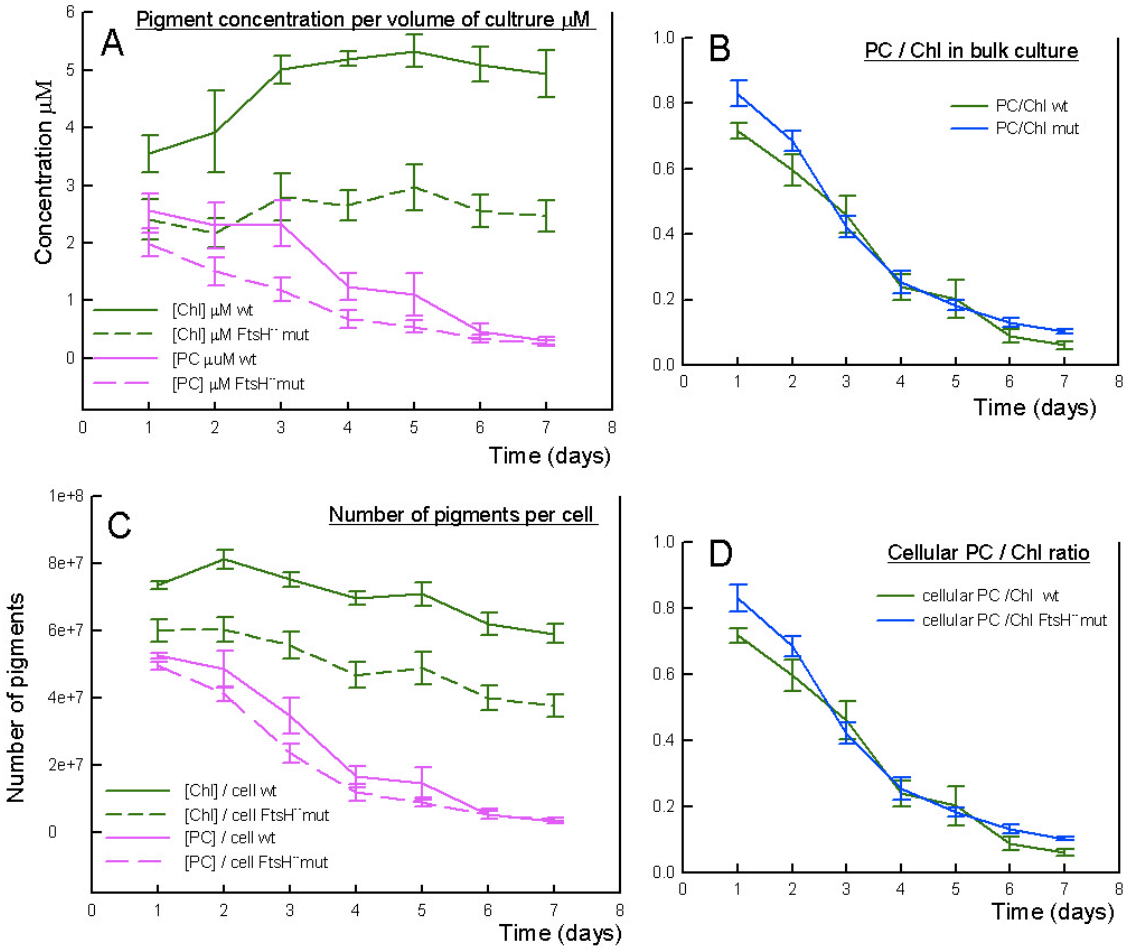

Figure V.11. **Chlorophyll and Phycocyanin concentrations during N-starvation. A)** Concentrations of chlorophyll and PC per volume of culture in µM. **B**) The ration PC / Chl in bulk culture. **C**) Cellular content of Chlorophyll and PC. **D**) Cellular ration PC / Chl . The error bars represent the standard error and are result of three replicants.

Pigment analyses of PC & Chlorophyll, have shown that both wt and FtsH$^-$ mutant appear to loose their phycocyanin with similar rate (Figure V.11.), regardless of any differences in their photosynthetic apparatuses.

The RT, whole cell absorption spectra, revealed an intriguing finding, viz. the loss of the overall cellular concentration of PB, Figure V.12. Comparing the ratio of absorbance at 625 nm to 680nm (phycobilins to chlorophyll), in whole cells, suggests that although both strains lose their phycobilins, for some reasons, the FtsH$^-$ mutant cells exhibited a characteristically faster rate compared to that of wt. It appears thus that during the N-limited growth the mutant cells, for some reasons resort to faster PBS degradation that the wt. Is this a random effect, a mere defect or it has something to do with the lack of FtsH protease and the repair mechanism?

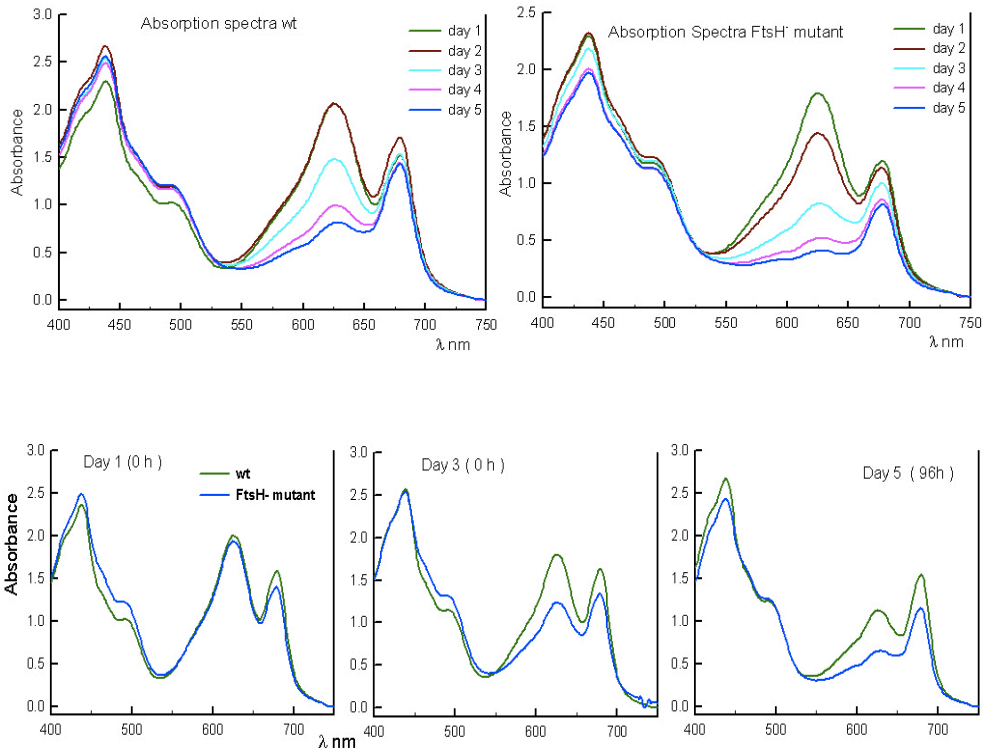

Figure V.12. **Whole cell absorption spectra at room temperature during N-starvation.** The same pattern appeared in all three replicants of the experiment. The top two graphs shows the absorption spectra in each type of cells separately, whereas the bottom three compare the Absorptions spectra between wt and FtsH$^-$ mutant.

6.2. Photosystem-II under nitrogen limited growth

As shown above, Figure V.1, FtsH$^-$ mutant exhibited characteristically higher F_{PSII} bands (685 & 695) than the wt, with the ratio of relative fluorescence yield of F_{PSI} / F_{PSII} to be

significantly reduced in the mutant (1.9 ±0.13 in wt to 1.03 ±0.17 in mutant). This was ascribed to larger proportion of light-inactivated PS-II centres because of a slower rate of D1 turnover in FtsH$^-$ mutant. The same pattern of fluorescence emission at 77K appears during nitrogen-limited growth, Figure V.13. The PS-II bands in FtsH$^-$ mutant are consistently higher than in wt for the duration of the experiment and the F_{PSI}/F_{PSII} ratio is reduced even further (<1), indicating thus that the overall concentration of PS-II in mutant cells remains higher than in wt. In this case however, namely, under conditions of nitrogen-deficiency, the larger overall population of photosystems-II cannot be attributed as easily to lack of FtsH protease because deprivation of cells from this essential element has been shown to lead strains of *Prochlorococcus marinus* to formation of non-functional PS-II reaction centres (Steglich *et al.,* 2001). Exposing the aforementioned cyanobacterium and yet the eukaryotic alga *Phaeodactylum tricornutum* (Geider *et al.,* 1993) to nitrogen limited growth, triggered the formation of non-functional PS-II centres in these species because of significant decrease in availability of newly synthesised D1 protein.

Although the reason for the accumulation of non-functional PS-II complexes in the above mentioned alga and cyanobacterium and in FtsH$^-$ mutant in our model organism seems to be an impeded repair mechanism, the causes however hindering the repair of photo-inactivated PS-II complexes in both cases are of different nature, i.e. decrease of D1 protein in *Prochlorococcus marinus* and *Phaeodactylum tricornutum* versus depleted proteolytical activity in FtsH$^-$ mutant in *Synechococcus* 7942.

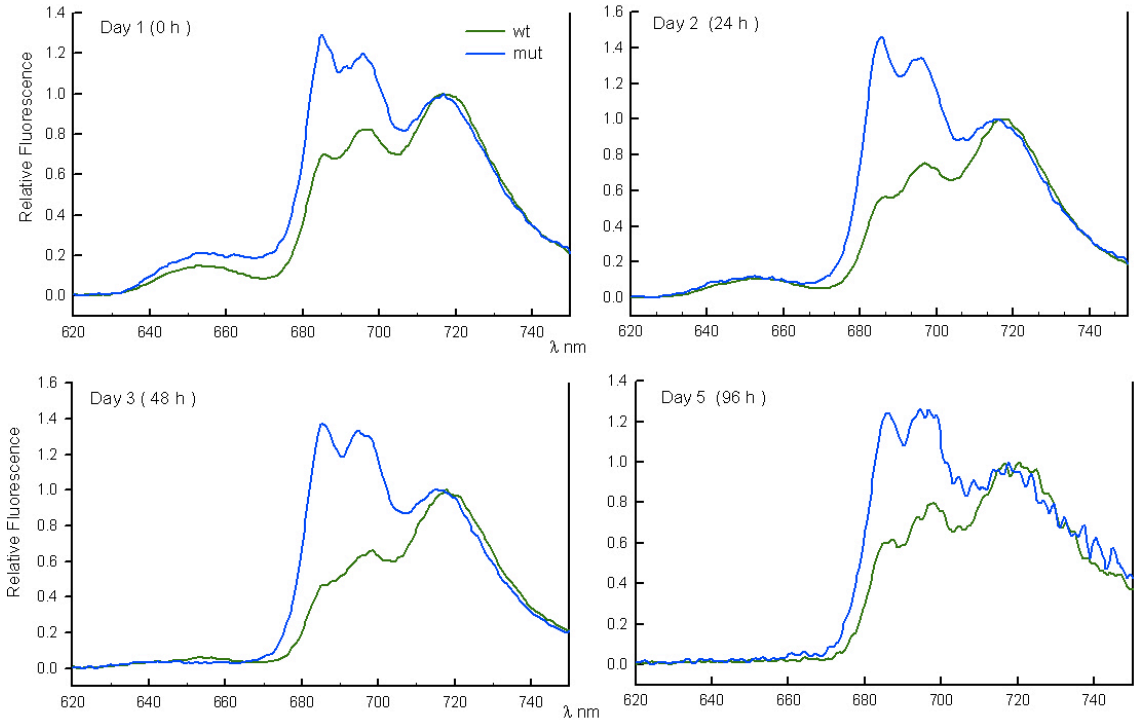

***Figure V.13*. 77K Fluorescence Emission during nitrogen deprivation.** Excitation at 435nm. The graphs are representative of three replicants. Prior to freezing in liquid nitrogen, all cells were dark adapted for five minutes, and the chlorophyll concentrations were adjusted to 5 μg ml^{-1}. The spectra are normalised to PS-I peak because the absolute amplitudes of fluorescence emission spectra at these conditions are unreliable. Worth mentioning that the signal after day 4 was invariably noisy.

To elucidate this issue we compared the 77K and RT spectroscopic data for the wild type and FtsH$^-$ mutant, Figure V.14., and yet quantified the content of PS-II centres during the nitrogen limited growth using radioactively labeled Atrazine (Table V.2.).

The low temperature emissions with excitation at 435 nm, show that while the overall content of PS-II decreases markedly in wild type cells (Figure V.14.A), in the mutant, despite the slight increase from the protruding peaks at 685 and 695nm (for more details on 685 nm band see below) the main bulk of PS-II fluorescence seems largely unchanged (Figure V.14.B). Consistent with these observations are the RT emission spectra. PS-II fluorescence in wt cells decreases (Figure V.14.C), whereas in FtsH$^-$ mutant appears to stay almost unaltered, (Figure V.14.D). Thus, the results from both RT & 77K spectroscopy show decrease of PS-II fluorescence in wt that remains principally unchanged in the mutant.

Quantification of PS-II content from binding of ^{14}C-labeled Atrazine, Table V.2., shows that the number of functional photosystems-II decreases in both wild type and mutant. It

is also noteworthy that this reduction is distinctly higher in wt (~49%) compared to that in mutant (~30%).

From the data above emerges that during N-starvation, the overall content, as well as the number of functional PS-II centres in wild type decreases, whereas in FtsH$^-$ mutant, with the overall number of functional PS-II remaining largely unchanged, it is only the number of functional PS-II that declines characteristically.

It becomes apparent that abiotic stress conditions, such as N-limited growth can induce not only degradation of light harvesting antennas in cyanobacteria but also can alter even further the stoichiometry of photosynthetic apparatus of the cell by reducing significantly the number of PS-II complexes. What triggers such response, viz., reduction of functional PS-II complexes when exposed to N-limited growth? There are two possible explanations for this. First, when under such stress, preservation rather than growth is apparently the first priority for the cell. Thus, being in high demand for this element, the cell blocks *de novo* synthesis of certain proteins amongst which is the D1 sub-unit. As a result, the repair mechanism of PS-II is severely restricted, leading inevitably to formation of non-functional complexes. This response has been described to operate in the aforementioned species of alga and cyanobacterium. Second, when under N-deficiency, and again with preservation being the first priority and not the growth, the cell may trigger a degradation process of PS-II centres as it does for phycobilisomes. After all, these are large multiprotein complexes that under extreme condition can be recycled for the preservation of the cell.

We favour the second possibility that the FtsH proteases may be involved in the degradation of functional PS-II complexes as well, given that the conditions require such extreme respond. Unlike *Prochlorococcus marinus*, no significant changes in the level of D1 protein have been reported in other cyanobacteria when grown under conditions of N-deficiency. In *Synechococcus sp* PCC 6301, formerly known as *Anacystis nidulans* as also our model organism *Synechococcus* 7942 and therefore often regarded as nearly identical species (Golden 1989), no notable decrease in D1 levels have been reported when starved from nitrogen (Biswal *et al.,* 1994).

Although loss of PBS appears to be a general response to nutrient limited growth (Reithman *et al.,* 1988) limitation of essential nutrients such as nitrogen and phosphorus, may eventually trigger distinct and independent responses in various organisms especially if these organisms have evolved under markedly different selective

pressures. *Prochlorococcus marinus,* commonly found in chronically low-nitrogen waters, is exclusively low light marine cyanobacterium (Partensky, *et al*.,1999 a, b), which seems to have evolved under one selective pressure, the vertical distribution of light and nutrients since its isolation is a function only of depth of isolation and not of the geographical latitude. In high contrast, freshwater *Synechoccocus* strains, predecessors of our model organism *Synechoccocus* 7942 (Wentzel 1989), can dominate equally well, both deep and shallow lake waters (Hauschild *et al.,* 1991, Vörös *et al.,* 1998; Callieri *et al.,* 2007), and most importantly are likely to endure P deprivation (Wentzel 1989) rather than N, which is most common limiting factor in marine environments (Yamanaka & Glaze*r 1980;* Wyman *et al.,* 1985; Yeh *et al., 1986)*

If the observed formation of non-functional PS-II in wild type and the FtsH$^-$ mutant is due to limited availability of D1 protein as in *P. marinus,* then, given that the rate of photo-induced damage is expected to be the same for both strains, the decline in the number of PS-II should be of equal proportions. The quantification results of PS-II with ^{14}C-labeled Atrazine argue against that. The measured reduction in wt (~49%) is considerably higher than in the mutant (~31%). Furthermore, if the hypothesis of reduced levels of D1 being the case, then the number of non-functional PS-II should be expected to rise and this increase to be manifested in fluorescence spectroscopy, most notably in FtsH$^-$ mutant. Again, neither 77K nor RT emission spectra provide such evidence, Figure V.14. While the decline of the overall concentration of PS-II in wt can be explained with the activity of FtsH proteases, the practically unchanged PS-II fluorescence yield in the mutant is against the hypothesis. Lack of D1 protein in the cells, especially in the mutant, should inevitably lead to increased PS-II fluorescence in relative proportion to the decrease (~31%) of PS-II as assayed with radioactively labeled Atrazine.

	Day 1	**Day 5**	**Decrease %**
Wild Type	$1.84*10^5$ ±15000	$9.6 * 10^4$ ± 4300	~ 49%
FtsH-mutant	$9.5 *10^4$ ± 5200	$6.6*10^4$ ± 5000	~ 31%

Table V.2. **Assessment of cellular PS-II content during N-limited growth.**

For all these reasons we favour the second possibility, namely that 360FtsH protease may be, directly or indirectly, involved in the degradation of functional PS-II centres along with its role in the repair mechanism. Under limited availability of nitrogen,

which fresh water *Synechococcus* strains are less likely to encounter in their natural environments, a more severe response may be elicited. Although there has not been an unambiguous proof that cells of *Synechococcus* 7942 growing in N-free medium enter a "dormant state", it has been nevertheless reported that small percentage of the cells can survive in this state for weeks (Collier & Grossman 1992).

If however FtsH are to be involved in the degradation of functional PS-II then it is noteworthy that one or more of the remaining FtsH proteases are likely to be involved in this process for the reason that the impressive decrease by ~30% of functional PS-II centres in the mutant can hardly be explained. Furthermore, conformational changes are required in the structure of the hetero-oligomeric ring-like structure of FtsH proteases to recognise as their substrate the functional reaction centres.

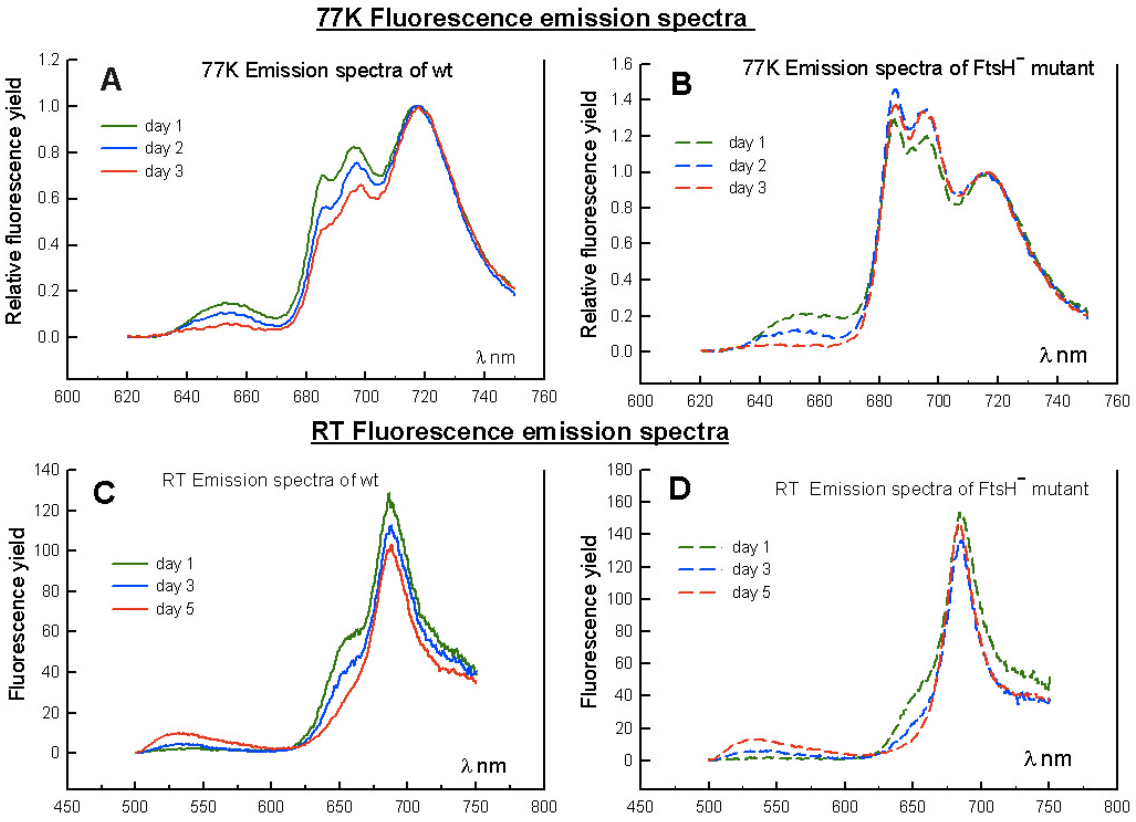

***Figure V.14* . 77K & RT Fluorescence Emission from wt and FtsH-mutant during N-deprivation.** A) 77K emission spectra with excitation at 435 nm in wt during N-starvation. B) 77K emission spectra with excitation at 435 nm in FtsH-Mutant. C) RT emission spectra with excitation at 435 nm in wt D) RT emission spectra at 435 nm excitation for FtsH-mutant.

The low temperature fluorescence emission spectra during N-limited growth showed yet another peculiarity observed almost exclusively during the course of the experiment (Figure V.13). The F_{685} peak that is believed to derive from CP43 core antenna, was characteristically protruding in all 77K recordings throughout the duration of the experiment. Yet, amongst numerous recordings of low temperature fluorescence (from cells growing in normal BG11), the same phenomenon occurred only twice and it is noteworthy that it was observed only in mutant cells.

Unusually high emission peak at 685nm and the simultaneous blue-shift of F_{PSI}, observed exclusively in $FtsH^-$ mutant, consisted a strong indication for the presence of IsiA protein in mutant cells. Presence of this chlorophyll binding protein, known also as CP43' due to its strong homology with the CP43 (PsbC) protein (Burnap *et al.,* 1993), has been linked with strong chlorophyll fluorescence at ~ 685 nm and with the blue shift of F_{PSI} peak (Pakrasi *et al*., 1985 a.b; Falk *et al* .,1995; Sandström *et al.,* 2001).

Ascribed initially to iron (Fe) deficiency, hence its name Iron Stress Inducible, CP43' has now been shown to be expressed under a multitude of conditions and therefore is believed to be involved in a number of different functions. Even though high transcription level is not always accompanied by protein synthesis, elevated expression of *isi*A gene can be observed under various conditions, e.g. light limited or high light growth (Foster *et al.,* 2006; Havaux *et al.,* 2005), oxidative stress (Li et al., 2004), heat & salt stress (Vinnemeier & Hagemann 1999), and even in some mutant cells such as in *Synechocystis* lacking cytochrome c_6 (Ardelean *et al*., 2002). The regulation of CP43' along with its structure (Burnap *et al.,* 1993) and ability to diffuse freely (Sarcina & Mullineaux 2004) in the membranes strongly suggest of specialised and yet flexible function for this protein. Undoubtedly, CP43' are associated with PS-I and form multimeric ring structures functioning as emergency antennas (Boekema *et al.,* 2001). Under certain conditions, a mobile population of this protein (Sarcina & Mullineaux 2004) can be associated with PS-II complexes quenching the excess of energy as heat (Park *et al.,* 1999; Sandström *et al.,* 2002; Sandström *et al.,* 2001) and may also be assisting the assembly of photosystems (Sherman & Sherman 1983).

Exposure of mutant cells, lacking the particular FtsH protease, to an additional stress such as growth in N-free medium, is plausible to have triggered the induction of this chlorophyll binding CP43' protein, either for attenuating the excitation pressure over PS-II or for assisting in the assembly of photosystems. In support to this proposal is the finding that during long-term growth of *Synechocystis* cells, the expression of *isi*A gene

exhibited a highly dynamic pattern and the only other gene that matched qualitatively this pattern was the *psb*A gene encoding for PS-II D1 core sub-unit (Singh & Sherman 2006).

6.3. Photosystem-II and light harvesting during nitrogen deprivation

The room temperature fluorescence emission, Figure V.15, performed simultaneously with the whole cell absorption spectra, correlated well with the data obtained from the latter and yet revealed some more interesting details. As both strains were cultured under dim fluorescent light and dark adapted before recording the fluorescence emissions, phycobilisomes may be expected to be associated mainly with PS-II.

The fluorescence spectra with excitation at 435nm clearly show that, while there is higher overall content of PS-II (F_{685}) in the mutant throughout the experiment (Figure V. 15.A), the PB level, seen as the band around 650nm, is yet again lower than in the wild type (Figure V.15.A). The observation is consistent with all fluorescence emissions at room temperature and the proposed idea of lower PBS content in the mutant.

Fluorescence yield with 600 nm excitation wavelength, Figure V.15.B, shows a strong excitation of phycobilisomes, peaks at ~650 nm, suggestin their correct association with PS-II complexes. The graphs in Figure V.15.B also reveal that the degradation of the light harvesting apparatus is characteristically faster in the mutant cells.

Quantifications of pigments chlorophyll-*a* & PC during N-starvation experiment have shown that no net chlorophyll degradation occurred in both types of strains. The marked drop in PC content, as per cell as per volume of culture, though expected, followed nearly the same pattern in both wt and $FtsH^-$ mutant. The results from the room temperature emission spectra with excitation at 600nm (Figure V.15.B) are consistent with the whole cell absorption spectra (Figure V.12.), and strongly suggest that the mutant cells, for some reasons, lose their PBS content faster than the wild type cells.

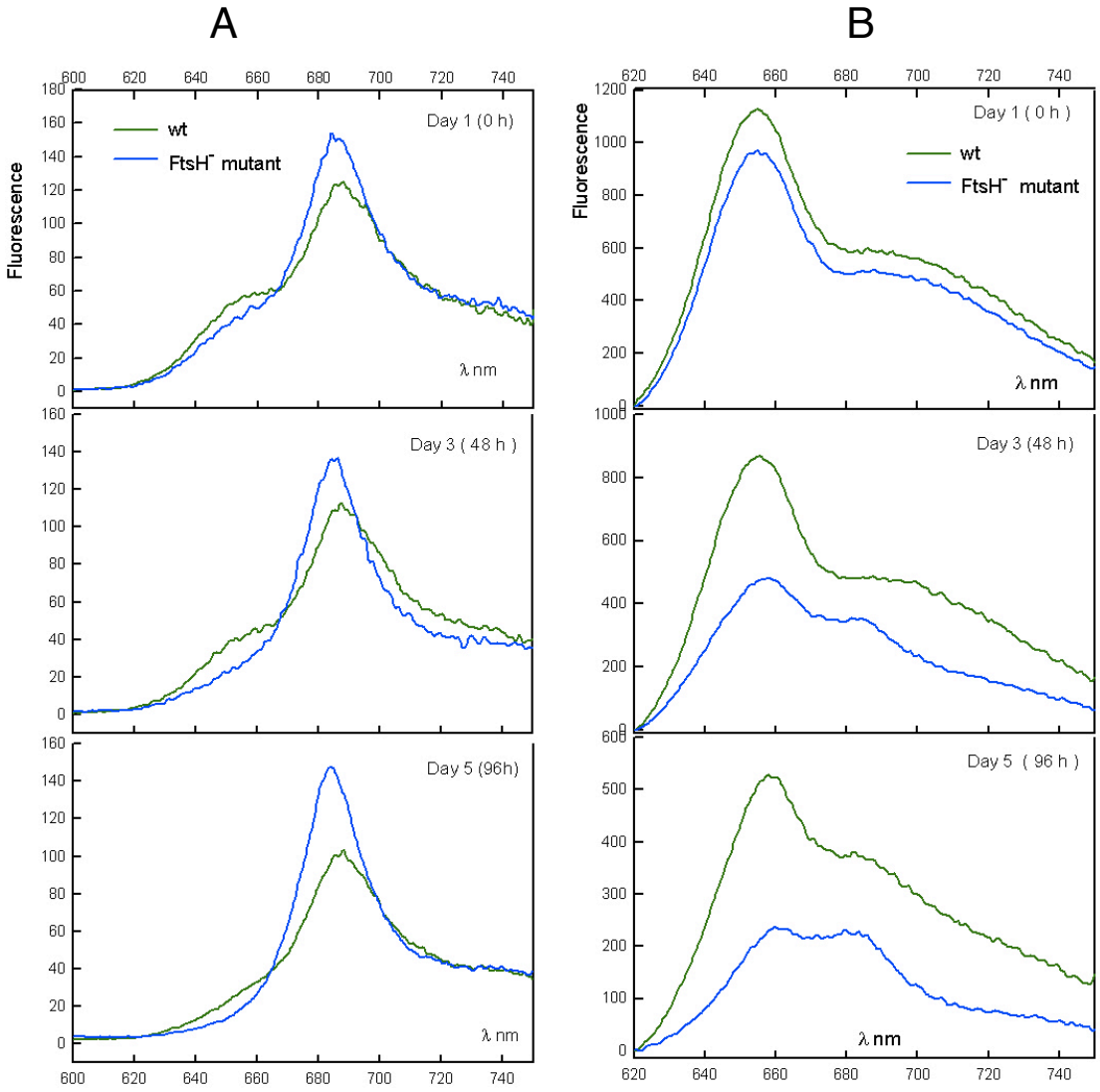

Figure V.15. **Room Temperature Emission Spectra during N starvation. A)** Excitation wavelength at 435. **B)** Excitation at 600nm. All data were adjusted to cell number.

Yet, the fluorescence emissions at 77K and RT provided a positive indication for a higher overall number of PS-II complexes in the mutant compared to that in wt. Combined together into one picture, all these findings show that the FtsH$^-$ mutant while contains more Photosystems-II than the wt, it appears to lose its light harvesting apparatus faster than the wt.

The question is, is this a random defect, or it is a response of the mutant cells to some kind of signal that dictates such response under the circumstances viz. N-deficiency and lack of FtsH protease?

The apparent fact of faster PBS degradation in FtsH$^-$ mutant than in wild type, itself implies that this particular protease does not appear to be involved in this process, namely the degradation of phycobilisomes during N-deprivation.

It has been known that the overall size of PBS antennas has a profound impact on the space between the thylakoid membranes (Westerman *et al.,* 1994; Olive, 1997). More recent studies (Collins *et al.,* 2012), using Hyperspectral Confocal Fluorescence Microscopy (HCFM) and Electron microscopy have revealed even more intriguing results on the effect of phycobilisome's size over the thylakoid membrane morphology, the distribution of photosystems I & II and the correlation between them. Using wild type *Synechocystis* 6803 cells and three genetically modified strains with different PBS, the size reduction of light harvesting was shown to have a profound effect on the large-scale morphology of TM; to increase the physical segregation of PS-I & PS-II; and most importantly to induce reduction of PSI/PSII ratio as a result of a simultaneous increase of PS-II centres and decline in PS-I numbers.

As the most plausible explanation to this inverse relationship between the size of PBS and the number of PS-II in TM, was that the cells modify their PSI/PSII ratio in order to compensate for the loss of excitation energy to PS-II because of the reduced size of PBS, thereby reduced light harvesting capability.

In our case however, this inverse relationship between the size of light harvesting apparatus, and the ratio of PSI/PSII appears as though it can be regulated from the other end, i.e., increased overall number of PS-II complexes in the mutant cell can bring about the reduction of light harvesting apparatus. Possibility that cannot be excluded when considering the plasticity of cyanobacterial cells and the fact that this inverse relationship between the photosystems and the size of PBS, was a result of a genetical mutagenesis to downsize the phycobilisomes in the cell of *Synechocystis* 6803 and the increased number of PS-II complexes in *Synechococcus* 7942 was also a result of genetical mutagenesis.

However, alteration of PSI/PSII ratio in *Synechocystis* 6803 due to deletion of *slr*0228 gene (Mann *et al.,* 2000; Silva *et al.,* 2003) did not trigger changes in spatial distribution of TM (Figure V.16.) as did the truncation of PBS (Collins *et al.,* 2012). In an early investigation whether the lack of FtsH protease from *Synechocystis* 6803 resulted in any changes or abnormalities in the large-scale morphology of TM, high

resolution Transmission Electron Microscopy images were obtained from wild type and the mutant grown under dim and hight light conditions.

If efficiency, best utilisation of light under particular conditions with minimal damages and energetic cost for the repair, is the quest for a cyanobacterial cell under any circumstances, then this inverse relationship between the light harvesting apparatus and the energy converters (reaction centres) is plausible and it may well be part of this great adaptability mechanism found in cyanobacteria. The above however is just a proposition, and though sound, there is apparently need for further research.

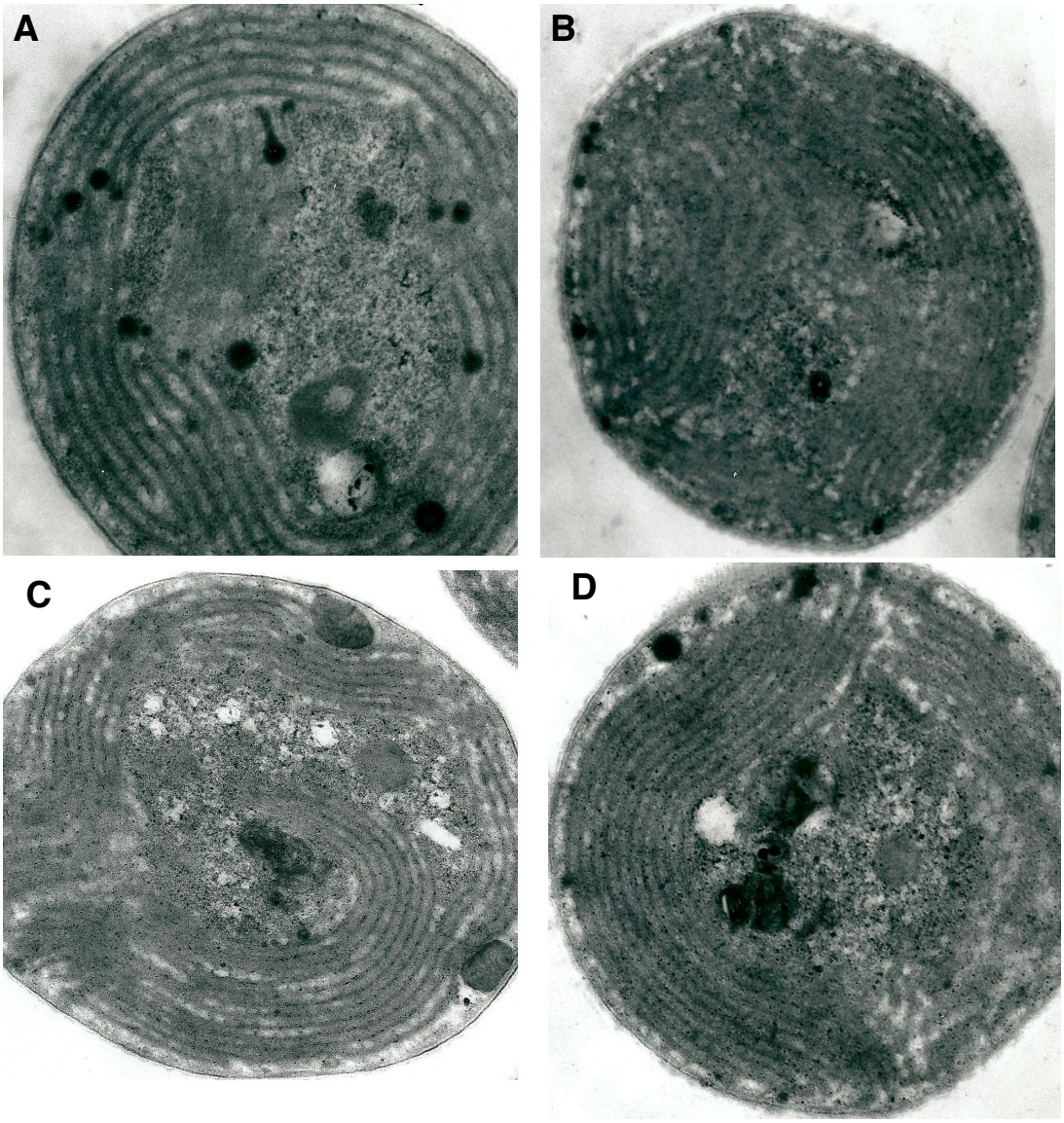

Figure V.16. **Transmission Electron Microscopy images from *Synechocystis* 6803 wild type and slr0228 FtsH-less mutant.**
Both types of cells were grown under dim (10 μmol. $m^{-2}.s^{-1}$) and high light (160 μmol. $m^{-2}.s^{-1}$) intensities. A) wild type grown under low light; B) mutant cells grown under low light; C) wild type grown under High light D) mutant grown under High Light.

Since interpretation of the data and not the acquisition of it, is often the most intriguing and fascinating part of the research, we suggest another possible explanation for the down-regulation of antennas in FtsH$^-$ mutant. This fast reduction of PBS in the mutant may be the result of two stress factors combined together, namely: lack of Nitrogen in the growth medium and the 360FtsH protein from cell's proteolytical machinery.

If 360FtsH protein is of major importance for the repair mechanism of PS-II, and the evidence thus far is in support of this theory, then cells deprived of the services of this protease will be under stress since their ability for fast removal of damaged D1 protein, and not only (Komenda *et al.,* 2006), will be seriously hindered. Under these circumstances and given the continuous inactivation of D1 protein at all light intensities, addition of another stress factor such as deprivation of cells from N, a major building material of proteins, looks likely to induce faster degradation of PBS in quest for nitrogen. Namely, an already impeded degradation of damaged D1 protein, now, under N-starvation, lacks the resources to synthesise the new D1 may be calling for more drastic measures and thus induces a faster degradation of PBS.

7. Dynamics of Thylakoid Membranes observed with Laser Confocal Microscope

Thylakoid membranes, as all biological structures, are dynamic systems, and Fluorescence Recovery After Photobleaching (FRAP) is now a well established technique, that provides the means to observe in real time the dynamics in these systems. Performed with a Laser Scanning Confocal Microscope (LSCM), this optical technique exploits the inherited ability of a laser to generate a coherent monochromatic light in an extremely narrow beam with an adjustable intensity.

With increased laser power, the fluorescent component under investigation is bleached over a small area of the membrane, and then, with the laser intensity reduced, the whole sample is scanned again and a series of images over a period of time are recorded. If the fluorophore in question is mobile, the fluorescence pattern of the sample will change with time, as the bleached area which is seen as dark will recover to an extent its fluorescence. However, in order to establish that the observed recovery of fluorescence in the bleached area is because of the diffusing chromophore, the possibility of reversible photobleaching of the chromophore must necessarily be excluded.

To obtain the diffusion coefficient of the component in interest, the fluorescence profiles along the long axis of the cell are extracted and the baseline fluorescence from pre-

bleached cells is subtracted. A Gaussian curve is then fitted to the corrected profiles. The fitting of the bell shaped curve will provide information about the depth and half width of the bleach at a series of time points, parameters that are used to calculate the diffusion coefficient (Mullineaux *et al.,* 1997; Mullineaux & Sarcina 2002; Mullineaux 2004)

Biological systems such as plasma membranes of eukaryotic cells are highly dynamic systems. Quantitative assays on ability to move within the membranes of various components show diffusion coefficients in the range of ~10^{-10} to 10^{-9} cm^2 s^{-1}(Zhang *et al.,* 1993). Any complexes diffusing at rates slower than 1-5 x 10^{-12} cm^2 s^{-1} are regarded as practically immobile (Zhang *et al.,* 1993).

Most of our current knowledge about photosynthetic apparatus is based on its static configuration and relatively little is known about the real time dynamics. In a quest to acquire an insight into this aspect of thylakoid membranes, with the use of FRAP, it was established that IsiA chlorophyll-binding protein for instance is mobile, and its diffusion coefficient was calculated to be (3.4 ± 0.8) x10^{-11} cm^2 s^{-1} (Sarcina & Mullineaux 2004). Even the bulky phycobilisomes have been shown to diffuse on the cytoplasmic side of TM of *Dactylococcopsis salina* (Mullineaux *et al.,* 1997) and *Synechococcus* 7942 (Sarcina *et al.,* 2001)

PS-II fluorescence cannot only be visualised with a Laser Scanning Confocal Microscope, but by deploying FRAP can also be assessed whether these complexes are confined more to a static configuration within TM or can diffuse under specific conditions (Sarcina & Mullineaux 2004; Sarcina *et al.,* 2006). To visualise PS-II, chlorophyll has to be excited with a light absorbed specifically by the pigment, combined with detection of this fluorescence in the far red region of the spectra. For the purpose, blue monochromatic light of 457nm from an argon laser is used to excite chlorophyll, followed by detection of emissions longer than 665 nm (Sarcina & Mullineaux 2004). As the immobilised on an agar surface cyanobacterial cells are kept throughout the experiment at their optimum growth temperature, viz., 30^{o}C, nearly 80% of the observed chlorophyll fluorescence under these conditions originates from PS-II (Mullineaux & Holzwarth 1993).

7.1. Light quality and PS-II configuration

Using FRAP it was shown that the diffusion coefficient of a major component of thylakoid membranes such as PS-II is below 2 x 10^{-13} cm^2 s^{-1}, (Table V.3) regarding

therefore the complex as virtually immobile, Figure V.17A (Sarcina & Mullineaux 2004). Taking into account the dynamics of photosynthetic membranes but most importantly the biological cycle of photosystem-II itself, the finding is very surprising. Being the most frequently damaged protein complex, and with piling up evidence that the repair of the system, in eukaryotic cells at least, is taking place in stromal lamellae, fact that implies the ability to move, confinement of PS-II to a rigid structural configuration is rather unforeseen. The fact not only raises questions about the reasons and the mechanism keeping the complex anchored but also consists an indication for *in situ* repair cycle in cyanobacteria at least, if not ubiquitous amongst photosynthetic organisms.

Nevertheless, PS-II does not always appear to be immobile since exposure of *Synechococcus* 7942 cells to different light conditions triggers biochemical changes in TM reversing temporarily the static distribution of the complex to a rather flexible configuration, Figure V.17B (Sarcina *et al.,* 2006). In particular, exposure of cells to monochromatic red light at 633nm from a Helium-Neon laser prior to FRAP analysis performed with blue light at 457 nm, induces not only mobilisation of photosystems-II but even redistribution of these complexes to certain areas along the long axis of the cell (Sarcina *et al.,* 2006). It is important however to emphasise that the reciprocity between the time and intensity of illumination with red light at inducing mobilisation, and much more redistribution of the complexes, is indicative that the effect, to an extent, is a red light dosage effect. Yet, mobilisation of PS-II is not the direct result of photodamage, as exposure of one end of the cell to red light is sufficient to trigger mobilisation of the complex at the other end of the cell (Sarcina *et al.,* 2006) suggesting also that the mobilised population of PS-II is not exclusively composed of damaged reaction centres.

Type of cells	Red Light treatment before FRAP	Diffusion coefficients	Source
wt	No	$< 2 \times 10^{-13}$ cm^{-2} s^{-1}	Sarcina & Mullinaux 2004
wt	Yes	$(2.3 \pm 0.4) \times 10^{-10}$ cm^{-2} s^{-1}	Sarcina *et al.,* 2006
FtsH^{-} mutant	No	$(9.8 \pm 3.2) \times 10^{-12}$ cm^{-2} s^{-1}	This study
FtsH^{-} mutant	Yes	$(8.6 \pm 3.2) \times 10^{-11}$ cm^{-2} s^{-1}	This study

***Table V.3*. Diffusion coefficients of PS-II in wild type and mutant cells, assayed after FRAP analysis.** The diffusion coefficients of PS-II in each type of cells were evaluated under two different conditions, namely with and without red light pretreatment**.**

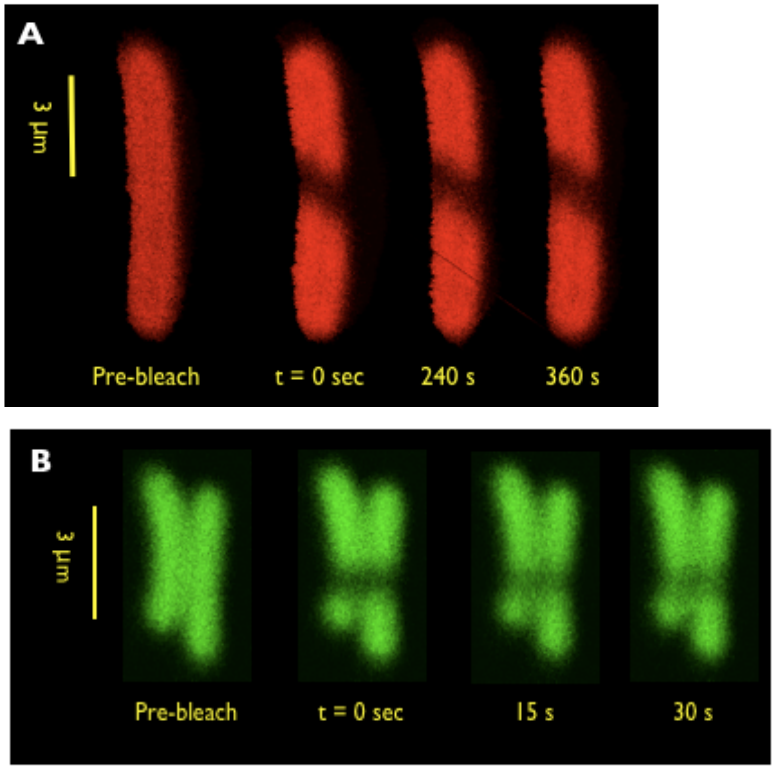

***Figure V.17*. Exploring the dynamics of PS-II with a Laser Scanning Microscope using FRAP.** A) FRAP analysis on PS-II mobility in *Synechococcus* 7942 without Red Light pretreatment. For details see Sarcina & Mullineaux 2004. B) Prior to FRAP, cells were treated with red light from an Helium-Neon laser. For more details see Sarcina *et al.,* 2006.

It appears thus that the flexibility of PS-II configuration within TM is contingent to certain degree upon the light quality and the amount of photons received from the particular spectrum. Yet, it is not the direct result of photodamage. Red light, contrary to blue, for some reasons is inducing mobilisation of the complexes and eventually redistribution after prolonged illumination. After all, these reaction centres are not always rigidly distributed, and there is at least one switch, red light, that can turn on, although temporarily, the mobilisation of these complexes.

The fact that PS-II complexes is normally found in rigid distribution, but can easily switch to a flexible state, given the appearance of the right stimulus(i), is a great finding providing vital insights into the real time dynamics of TM. Nevertheless, as it is often the case in scientific research where finding the answer to one question give rise to several others, these results alike raised some other interesting questions, e.g., why this wavelength specificity, what is the mechanism triggering these changes and what is the biological significance of it, if any?

Although this particular effect of red light is not the direct result of photodamage, nonetheless the possibility whether it is due to the damaging severity of this light had to be tested. Is red light more effective at inducing photoinhibition that other lights? To gain an insight into this aspect, a comparison of the damaging effects on PS-II activity for different light spectra was performed by recording the oxygen evolution under photoinhibitory conditions for each spectrum, Figure V.18. The presence of Lincomycin in the media during the experiment ensured absolute absence of the repair mechanism, and therefore these measurements along with oxygen evolution, also reported on PS-II degradation rates. The results, Figure V.18, show that all lights: red, blue and white, have nearly the same inhibitory effect, excluding thus the possibility that the mobilisation of PS-II by red light is associated specifically with the damaging effect of this light.

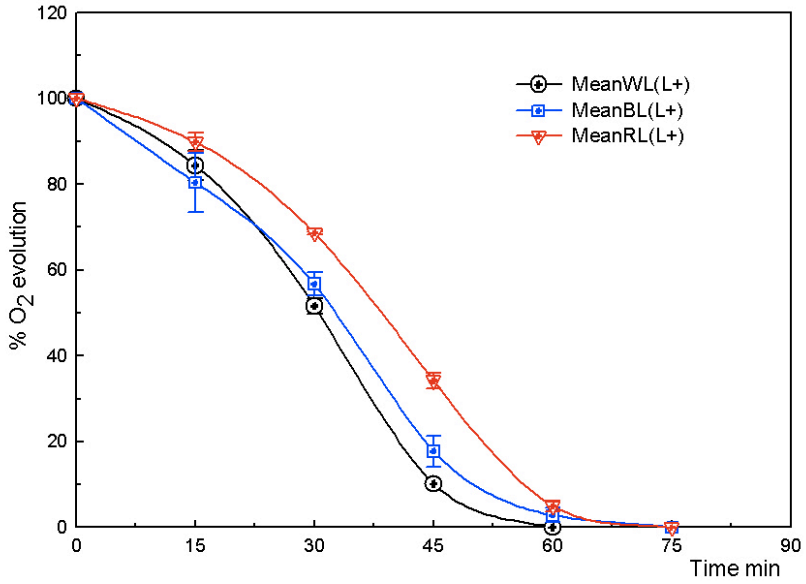

Figure V.18. **Testing the damaging effect of blue, red and white lights on PS-II activity.** The impact of Red, Blue and white lights on PS-II performance was assessed by recording the oxygen evolution in the presence of Lincomycin. In each case the intensity of light was adjusted to 2000 μE m^{-2} s^{-1}. As no electron acceptors were added during the experiment the presented results are from the entire electron transport chain. In all cases, oxygen evolution is presented as relative rate, viz. in proportion to the rate prior to photoinhibition.

In addition to the above, a large series of oxygen evolution measurements, performed for each light spectrum with and without lincomycin, led to some thought-provoking results. As mentioned earlier on in this chapter, comparison of oxygen evolution rates in the presence and absence of the aforementioned antibiotic, provides important information about the activity of the repair cycle. Thus, when these rates for each light spectrum were plotted together, the comparison showed that although both lights are equally damaging, Figure V.18, the repair cycle under red light is by far more responsive than with blue, Figure V.19A,B. Under the latter it becomes operationally recordable only after ~20 minutes, Figure V.19A. In contrast to blue light where during the first 15-20 minutes the oxygen evolution decreases at nearly equal rates with and without lincomycin, Figure V.19A., under red light for the same period, with the repair cycle unimpeded (lack of lincomycin) there is significant increase in oxygen evolution, Figure V.19B. The results show that while the appearance of red light launches an alert state in the cell with the repair cycle operating immediately at high rates, the same fluency of blue light leaves the cell for nearly 20 minutes somehow ‘unconcerned’, time however sufficient to have caused considerable amount of damage.

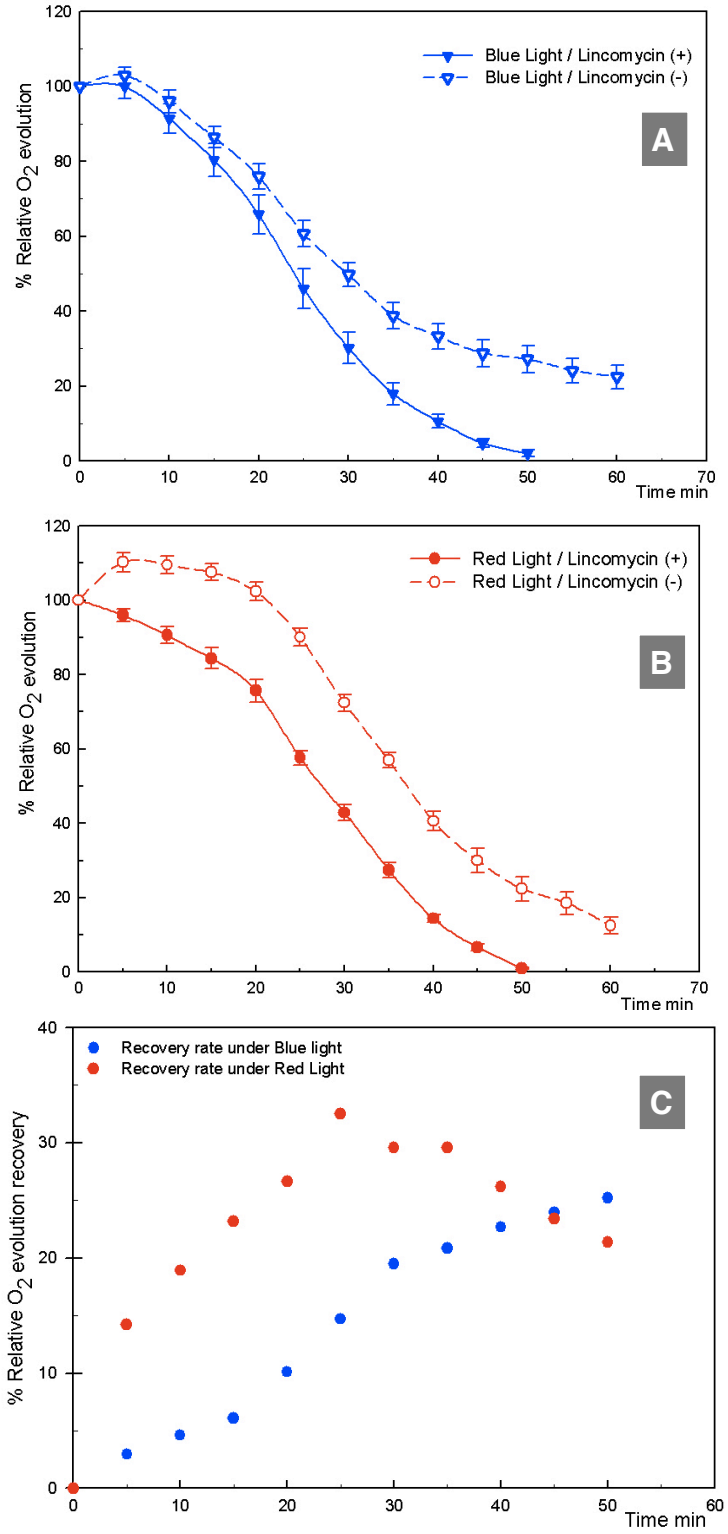

Figure V.19. Photoinhibition and Repair Cycle of *Synechococcus* 7942 as recorded by Oxygen evolution. The rates for each light, red and blue, were assessed under photoinhibitory conditions, 2000 µE cm^{-2} s^{-1}, and in the presence and absence of Lincomycin. The results are presented as relative rates and report on the entire electron transport chain as no electron acceptors were used. A) Rates under Red Light with and without Lincomycin. B) under Blue light with and without Lincomycin C) Activity of the repair mechanism under red and blue lights. The graphs for red & blue lights were constructed by subtracting the data of O_2-evolution with lincomycin, from that without.

From Figure V.19C., displaying the activity of the repair mechanism under each light, this difference in timing for launching the repair cycle at high rates becomes more evident. Because of this contrast in timing, the trigger for the repair cycle for each wavelength spectrum is believed to be of different nature. There is an immediate and sharp increase in the activity of the repair mechanism under red light, culminating after 25-30 minutes of activity, Figure V.19 C. This is not seen under blue light. Under the latter, a considerable increase in the activity of the repair cycle commences only after 15 minutes of action, Figure V.19C. The instantaneous response of the repair cycle to red light indicates that the red light itself is perceived as a signal capable of launching the mechanism. Although the particular photoreceptor is unknown, it is likely to be a phytochrome-like molecule (Sarcina *et al.,* 2006). Such far-red and red-light photoreceptors are no longer believed to be exclusively found in plants, as several of them have been identified and characterised in cyanobacteria, including *Synechococcus* 7942 (Yeh *et al.,* 1997; Los *et al.,* 2010; Mutsuda *et al.,* 2003). In contrast, the late switch of the cycle under blue light, suggests that the signal for its commencement is of very different nature. The amount of photoinactivated PS-II complexes during this time may have caused changes in the redox state of electron transport chain components. Thus the redox state of these co-factors is likely to function as a signal transduction pathway, increasing ultimately the activity of the repair cycle. Unlike the phytochrome-triggered reactions, the response of the electron transport chain as signal transducer, is not exclusively contingent upon light (quality and / or quantity) but also upon a series of other factors, e.g. time, temperature, current matabolic demands, that in turn affect the oxidative state of the electron transport chain (reviewed by Mullineaux 2001). The fact of the entire electron transport chain functioning as a 'photoreceptor' is not unknown to cyanobacteria (Fujita *et al.,* 1996). Consistent also with the hypothesis is the fact that no blue light specific photoreceptors have been found in bacteria, apart from Cph2 that is believed to play possibly a role in blue-light signalling (Braatasch & Klug 2004).

The fact that the red light triggers simultaneously mobilisation of PS-II complexes and their Repair Cycle is a clear indication that the latter two phenomena, viz., mobilisation & repair must be inherently associated. Yet, as mobilisation of PS-II occurs at high intensities of red light, when the photo-induced damage is expected to be high, then mobilisation of PS-II appears to facilitate somehow the repair cycle.

7.2. Light quality and PS-II repair cycle

Despite the progress of recent years in understanding the repair cycle of PS-II, a good number of its aspects remains, to one or another degree, elusive. One such issue is the site of the repair. Is it *in situ* or in specially allocated zones referred to as repair zones? Our current knowledge is not only gleaned largely from *in vitro* studies but it is also based on a rather static configuration of photosynthetic apparatus and seldom is supported with *in vivo* data reflecting the real time dynamics within TM. Besides, looking for an unambiguous answer to the above question, as it is often the case, looks rather as a conventional approach since it takes into consideration very little, if any at all, not only the great plasticity of photosynthetic organisms but often the evolutional background of the model organism itself. Yet, isn't this very interference of the organism with its environment, the ever changing conditions, that results in emergence of new features, and stabilisation and reinforcement of others? This magnificent plasticity of cyanobacteria that made them so successful for billions of years is based on their great adaptability to the ever changing conditions, as seasonal as those over the lifespan of an individual cell. Taking all that into account and yet the light cycle over 24 hours, from virtually zero to a few thousands of μE m^{-2} s^{-1}, the view that a photosynthetic organism would have evolved and conserved just one repair mechanism, as far as the repair site of it is concerned, seems rather unrealistic. Congruous with all that and above all with the primary aim of survival for each organism, is the fact that the environmental conditions, mainly intensity and quality of light, will dictate what is required more from the repair cycle, effectiveness or energetic cost.

The low intensity light used for growth of cells and the blue light used subsequently for FRAP analysis seem to keep PS-II in a rather rigid configuration. Even illumination of bulk culture with bright blue light of 2000 μE m^{-2} s^{-1} or exposure of cells to extremely high intensities generated by the Laser Scanning Confocal Microscope before FRAP, have no effect on PS-II mobility (Sarcina *et al.,* 2006). The fact that under blue light Photosystem-II remains anchored (Sarcina & Mullineaux 2004) while simultaneously being damaged, Figure V.18, consists a strong indication that the repair of the complex under these condition, low light and / or predominance of blue wavelengths, must occur *in situ*. In high contrast, in the presence of red light, mobilising up to 50% of the complexes (Sarcina *et al.*, 2006), the repair of these reaction centres is likely to take place *in situ* and / or in the repair zones.

With all these in mind, the question seeking now an answer is: why this wavelength specificity? What is the biological significance of such response? Does it furnish the cell with an adaptational advantage or it is merely a random response?

The depth of penetration of light into water, although substantially affected by the 'turbidity' of the water viz., amount of silt, detritus materials, and dissolved minerals, it is essentially a function of light's wavelength. The general principle is that the shorter the wavelength the deeper it penetrates into the water and vice versa. Red light *ipso facto* can only be found in the shallows or near the surface, while blue-light in contrast, consists the only source of light in deeper layers of the euphotic zone.

The freshwater *Synechococcus* species, including our motile strain PCC 7942, can be commonly found as near the surface as in deep waters (Hauschild *et al.,* 1991, Vörös *et al.,* 1998; Callieri *et al.,* 2007).

In natural environments, the condition of blue light predominance can only be repeated in deep waters where the overall intensity of light is greatly reduced. This reduced irradiance may allow the cell to adopt a 'relaxed' *modus operandi* for the repair cycle, because under these conditions and with low rates not only of photoinactivation but photosynthesis as well, the energetic cost of the repair must be of more essence for the cell than the effectiveness of the mechanism. The observation is consistent with the fact that repair mechanism under blue light photoinhibitory conditions, Figure V.19A., is hardly noticeable for nearly 15-20 minutes, time however sufficient to cause a substantial amount of damage under such intensities if occurs regularly. This noticeable insensitivity of the cells to blue-light-damage to PS-II, is indicative that there is no particular need for such high performance repair cycle under these conditions as they are unlikely to occur in nature for the obvious reasons mentioned above. Nevertheless the slow response of the repair mechanism under particular conditions may be because the cells undertake different measures to counteract the problem. Blue light has been shown to initiate a rapid exchange of D1:1 with D1:2 (Tsinoremas *et al.,* 1994) that in turn helps to overcome the problem by faster D1 turnover and by improving the intrinsic photochemical efficiency of PS-II. In contrast, red light not only induces mobilisation of PS-II, Figure V.17B., but also a very responsive and effective repair mechanism, Figure V.19B. These experimental observations correlate also well with the fact that the red spectrum can only be found near the surface of the euphotic zone where the overall intensity of light is high and so is expected to be the rate of photoinduced damage. Nevertheless, being a red light-dosage effect, mobilisation of PS-II cannot be

triggered by the mere presence of red light but only when the right amount of photons of certain energy will be received, or in other words, under conditions of intense sunshine. Thus floating for long hours under sunlight, more 'drastic' measures, where effectiveness and not the energetic cost, may be required to cope with the increased rate of photo-inactivation. From this point of view, mobilisation of PS-II, triggered by the red light, is plausible to be a response facilitating photoprotection. Given the need for effective and fast repair cycle under high irradiance, when other subunits along with D1 may need to be replaced as well, it is likely that the mobilisation of the RC is to increase the efficiency of the repair mechanism by transporting the damaged population of PS-II to 'industrial' zones, the repair zones. In the view of abundant availability of energy, light, the energetic cost is not any longer of prime importance but a quick and effective repair instead.

7.3. FtsH protease and PS-II configuration

From the accumulated data emerges that light quality has effectively an impact on the status of PS-II configuration as well as on the responsiveness of the repair cycle. Yet, the signalling mechanism triggering the latter appears to be of different nature for red and blue lights, viz., photoperception vs redox state respectively. Because mobilisation and repair cycle of PS-II are both induced by the same stimulus, red light, these two phenomena by inductive reasoning must be associated. In other words, the mobilisation of PS-II is believed to facilitate, in one or another way, the repair cycle or the overall photoprotection mechanism.

Furthermore, the results of insertional mutagenesis of 360*fts*H ORF in *Synechococcus* 7942 indicate that this particular protease seems to be involved in the Repair cycle of PS-II and may possibly play a role in degradation of functional RC under N-stress. Mutant cells, when compared to wild type, also appear to have reduced number of functional PS-II reaction centres but with higher overall concentration of these complexes. Yet, from the general knowledge about the structure and *modus operandi* of these metaloproteases it seems that they are not confined to PS-II repair cycle but play a much broader role in quality control within TM.

Based on all that, a series of FRAP analysis was performed on FtsH^{-} mutant cells to investigate whether the lack of this protease from the proteolytical machinery of the cell would have an impact on PS-II dynamics. The study resulted in some very interesting findings.

In particular, FRAP analysis without the red light pretreatment showed that PS-II complexes in mutant cells, in contrast to their rigid configuration in wild type, are mobile, Figure V.20A and Table V.3. With the diffusion coefficient calculated at (9.8 ± 3.2) x 10^{-12} cm^{-2} s^{-1}, (Table V.3), these reaction centres in the mutant are classified as operationally mobile (Zhang *et al.,* 1993). Despite their slower motion compared to that in wild type when exposed to red light prior to FRAP, (2,3±0.4) x 10^{-10} cm^{-2} s^{-1}, (Sarcina *et al.,* 2006), their diffusion within TM is nevertheless considerably faster than that in wild type with no red light pretreatment, which are characterised as operationally immobile < 2 x 10^{-13} cm^{-2} s^{-1}, (Sarcina & Mullineaux 2004). It emerges consequently, that red light and lack of 360FtsH protease from the cell have the same impact on PS-II configuration, i.e., mobilisation of the RC.

Illumination of mutant cells with red light prior to FRAP, Figure V.20B., resulted in an increased diffusion coefficient of PS-II, (8.6 ± 3.2) x 10^{-11} cm^{-2} s^{-1}. This recorded increase of the diffusion coefficient is believed to be due to the synergy of an extrinsic factor, red light, with an intrinsic one, lack of 360FtsH protease from the cell. Nonetheless, none of these two factors, either separately or synergistically can induce mobilisation of the entire population of PS-II. Neither the increase of red light dosage (Sacina *et al.,* 2006) nor even the two factors combined together, Figure V.20C., can bring about such result. Mutant cells undergone a second FRAP analysis after 5 minutes of recovery and red light illumination, were unable to recover the fluorescence in the bleached area and exhibited a remarkable decrease in diffusion rates, Figure V.20C.

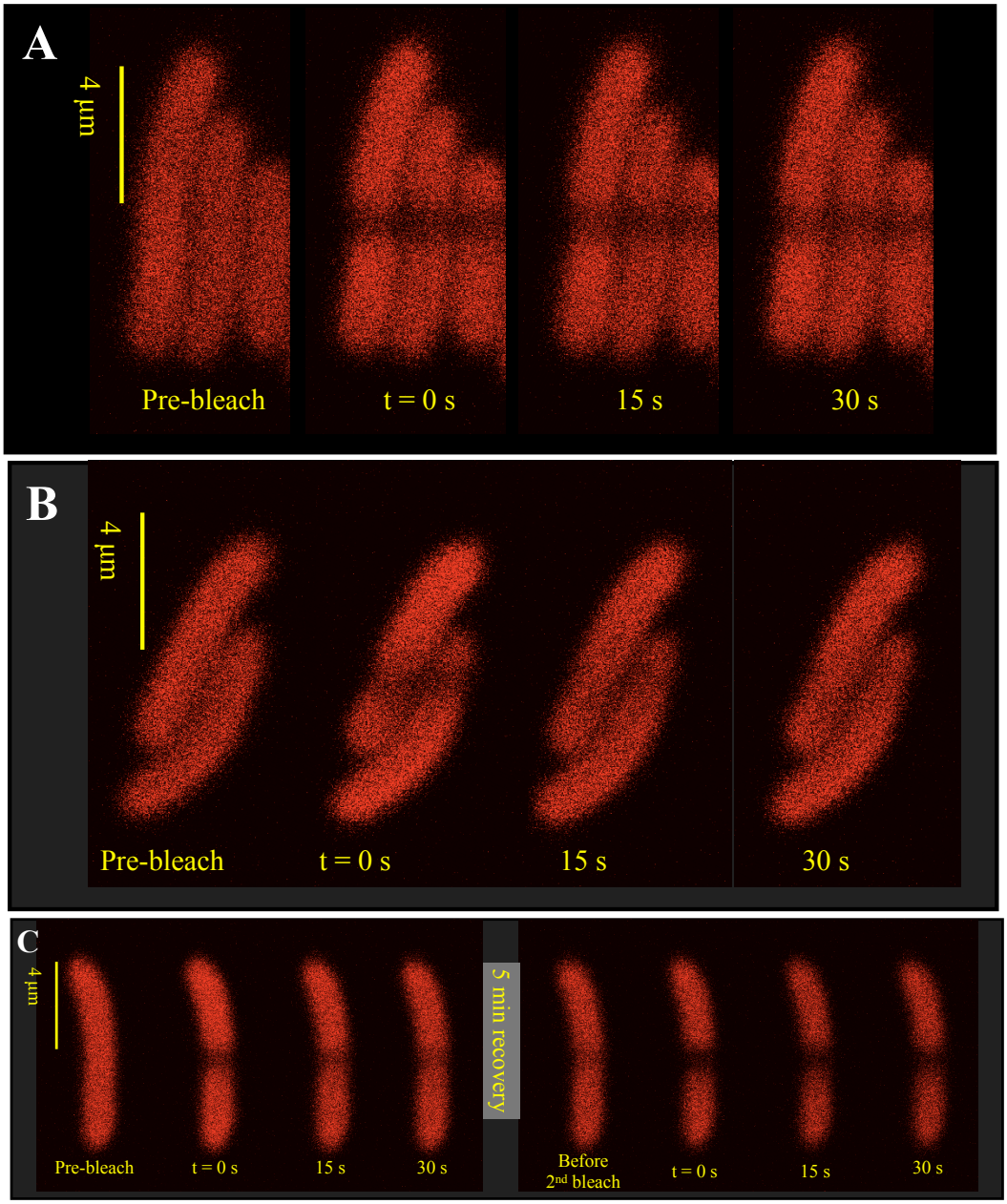

***Figure V.20*. Exploring the dynamics in thylakoid membranes of FtsH^{-} mutant. FRAP analysis on PS-II dynamics within thylakoid membranes.** A) Cells were not pretreated with red light before performing FRAP. B) Prior to FRAP, cells were exposed to red light. C) Cells pretreated with red light prior to FRAP analysis and then left for min to recover. After the period of recovery the cells, as previously, were again illuminated with red light before performing the second FRAP experiment.

In spite of this apparent similarity at inducing mobilisation of PS-II, the dosage of red light and the particular protease have effectively had opposite functional roles. As the dosage of red light is the prerequisite for PS-II mobilisation, FtsH alike is the prerequisite for keeping PS-II anchored, since lack of this protease, and not the presence of it results in flexible configuration of the complex. Therefore, as far as mobilisation of PS-II is concerned, there must be a very different reason for it in each case. Red light,

capable of mobilising only up to 50% of the overall PS-II population in wild type, is believed to facilitate somehow the repair cycle of the reaction centres by alerting the cell for the forthcoming high light intensities near the surface of the open waters in the natural environment of our model organism. The fraction of mobile PS-II complexes in this case seems to be composed largely of functional reaction centres along with non-functional ones. Illumination of one end of the cell with red light and mobilisation of PS-II at the other end of it, observed after FRAP analysis (Sarcina *et al.,* 2006) consists a strong indication for the above mentioned proposal. The use of wild type cells (repair cycle intact), grown under normal light intensities (thereby lower rates of photoinduced damage) are all in favour of this idea.

Contrary to red light, FtsH protease, which can keep only certain amount of PS-II anchored, is an essential part of this repair cycle and therefore is believed to prevent mobilisation of damaged and / or misassembled PS-II mainly, if not exclusively. The *A. thaliana* FtsH2 (VAR2) has been suggested to be involved in translocation events because of the high similarity it exhibits towards the red pepper Pftf protease (plastid fusion/protein translocation factor) a homologous protein from AAA family (Hugueney *et al*., 1995; Chen *et al*., 2000). Immobilisation of damaged RC by FtsH holoenzyme facilitates for apparent reasons the repair of PS-II. Considering the complexity of the cycle involving a legion of secondary processes such as monomerization of the complexes, partial disassembly, temporary storage of pigments and electron transport co-factors, insertion of new subunits, reassembly and others, along with the spatial configuration of thylakoid membranes it becomes obvious that the process of repair, whether it takes place *in situ* or in the repair zones, must be performed with the reaction centres immobilised. For those reasons, the mobile fraction of PS-II complexes, observed in $FtsH^-$ mutant, Figure V.20B, is believed to be composed, mainly if not exclusively of photoinactivated or / and misassembled reaction centres. Given the absence of this particular FtsH protein from the cell and an impeded therefore repair cycle, this considerable amount of non-functional PS-II complexes, is mobile and in a state of ‘confusion’, wandering in search for repair.

CHAPTER VI

COMPUTATIONAL ANALYSIS OF FtsH PROTEINS

Chapter VI
Computational analysis of FtsH proteins

1. Introduction

The ability of FtsH proteases to recognise as their substrate both soluble and membrane embedded polypeptides, either unfolded, non-assembled, misassembled or damaged, established them as an essential part of membranes' quality control mechanism (Akiyama 1994; Langer 2000). Yet, the strong sequence conservation, the almost universal distribution amongst living organisms, along with the manifestation of severe defects in FtsH-less mutants, in a wide range of organisms, are all facts verifying their functional importance and significance either for cell homeostasis or survival.

FtsH proteins are characterised by a markedly high degree of similarity at primary level. As members of 'AAA' protein superfamily, they are ATP-dependent proteases containing one AAA cassette (Walker motifs A & B, and the Second Region of Homology SRH), and yet are distinguished by the two transmembrane helices towards their amino termini, a leucine coiled coil and zinc-binding site at their carboxyl termini.

The extreme sequence conservation at one hand side and the wide range of activities these proteases are associated with at the other, consist, probably the most paradox characteristic of this family. There seems to be a significant reason for sequence conservation as among FtsH proteins with common function in different species (orthologs) as among the same proteins performing a number of different functions within one species (paralogs).

The evolutionary history of these AAA metalloproteases is not well understood. However, in contrast to bacteria, that in principle appear to contain a single copy of *fts*H gene, in cyanobacteria and to larger extent in green plants, FtsH-proteases are encoded by a multigene families (for details see below). Although the functional significance of having more than one of these metalloproteases is not as yet fully understood, it is believed that it stems from the *modus operandi* of the holoenzyme that in its operational form constitute a hetero-oligomeric ring-like structure (Kihara *et al.,*1996; Shotland *et al.,* 1997; Kihara *et al.,* 1998; Karata *et al.,* 1999). In plants, it requires the presence of two different types of FtsHases (Sakamoto *et al.,* 2003; Sakamoto 2003) that are not interchangeable (Yu *et al.,* 2004; Zaltsman *et al*., 2005; Yu *et al*., 2005). It is probably this hetero-oligomeric nature of the functional enzyme that may explain the multifunctionality of FtsH-proteins, with the function it is required to perform

regulating its stoichiometry. Most of these polypeptides in plants appear to exist in pairs, encoded by closely related genes (Yu *et al.,* 2004). To explain the leaf-variegation in FtsH-less mutants in *A. thaliana* the 'threshold model' has been proposed (Yu *et al.* 2004). According to this model, slight differentiation in gene expression of either type in different cells, pushes the concentrations of the remaining FtsH proteins, either below or above the 'threshold' that is the requirement for accumulation of functional holoenzyme and hence the development of normal chloroplasts (Yu *et al.* 2004; Yu *et al.,* 2005)

If the attainment of 'threshold' concentrations of enzyme's subunits is essential for its proper functionality, then duplicating the number of gene copies at first place may have been important during evolution, i.e increased number of gene copies, increased levels of expression given the involvement of these proteases in legions of regulatory processes.

The evolutionary mechanism of gene duplication allows organisms to acquire redundant copies of genes. The redundant copies are then 'free' to evolve new functions and become homologous genes with different functions (paralogs). Multiplication of the FtsH proteases in cyanobacteria and to larger extent in higher plants seem to correlate with the evolution of oxygenic photosynthesis in cyanobacteria and with the evolution of chloroplasts in higher plants.

In this chapter we substantiate the reasons why we targeted particular *fts*H genes in *Thermosynechococcus elongatus* BP-1 and *Synechococcus* sp. PCC 7942 for knock-out mutagenesis. All FtsH protein sequences present in the aforementioned organisms along with those identified in *Synechocystis* 6803were aligned and a phylogenetic tree was constructed. Yet, the availability of hundreds, if not thousands of species with known genome sequences created an irresistible urge to investigate the distribution of FtsH proteins among living organisms. Thus, multitude of homology searches (BLAST) were performed in all phyla containing photosynthetic species, even as little as one, e.g. *Acidobacteria*. Furthermore, in order to understand better the relationships between FtsH-proteases in different species, multiple sequence alignments were performed and phylogenetic trees were constructed. Finally, to gain an insight into common features of orthologous FtsH proteins, structural analyses were also performed.

2. Selecting cyanobacterial *fts*H genes for mutagenesis

Probably the most powerful idea in bioinformatics is that the history of a protein and the physiological role it will play in its mature form are imprinted to certain degree in its primary structure. Proteins that have derived from a common ancestral form tend to exhibit similarities in their amino acid sequences and yet the higher that similarity is the higher the probability that these proteins will be involved in the same physiological process(es). This fact exploited inimitably by modern computers with their immense calculating capabilities, consisted the bedrock not only for legions of new predictions and discoveries but also helped us to gain new, unheard of before insights into the evolutionary relationships among living organisms. The knowledge *ipso facto* of a protein's functional role, based on experimental data, combined with that of its primary structure, can be proved invaluable not only in predicting homologous proteins in other, even evolutionary distant organisms but also in identifying those structural characteristics defining the group of orthologous proteins.

In search for protease identity involved in the repair cycle of PS-II, *in vivo* studies with *Arabidopsis thaliana* and *Synechocystis sp.* 6803 have shown that an FtsH homologue seems to be involved in the early stages of D1 proteolytical degradation (Bailey *et al.,* 2002; Silva *et al.,* 2003). Given the availability of Genomic DNA sequences for other cyanobacterial species such as our model organisms *T. elongatus* BP1 and *Synechococcus sp*. 7942, and the sequences of the above mentioned FtsH homologues, namely FtsH2 and slr0228FtsH respectively, the next step almost inevitably was to investigate the proposed role for FtsH proteases in new species. And though the choice of *T. elongatus* BP1 and *Synechococcus sp*. 7942 was certainly based on availability of their genome sequences, the advantages these two organisms offer as model systems, especially for investigation of real time dynamics in TM, unquestionably played a major role as well.

The cyanobacterium *Synechocystis* sp 6803 contains four *fts*H ORFs: *slr*0228; *slr*1390; *slr*1640; and *sll*1463; (Kaneko *et al.,* 1996) encoding for four distinct forms of the protease, Table VI.1. All four of these genes were targeted with insertional mutagenesis (Mann *et al.,* 2000). Representatives clones carrying mutations in *slr*1390 and *slr*1604 ORFs exhibited incomplete segregation even after multiple rounds of re-streaking. Inability to isolate mutants that lack the wild type copy of these particular genes, suggested that these two particular genes, alike the *fts*H gene in *E. coli* (Tomoyasu *et al.,* 1995), are of immense importance for cell viability. In contrast, clones with mutated

*slr*0228 and *sll*1463 ORFs were fully segregated and though both dispensable, the *slr*0228FtsH, unlike *sll*1463, proved to be an essential element of TM quality control mechanism (Mann *et al.,* 2000; Silva et al., 2003; Komenda *et al.,* 2006).

Organism	Sequencing centre / Protein ID	Synonyms	NCBI Accession	Notes
Synechocystis 6803	Kazusa DNA Res.Ins. slr1390	**FtsH1** (Sokolenko *et al.,* 2002)	NP_440525.1	Indispensable (Mann *et al.*, 2000)
	slr0228	**FtsH2** (Sokolenko *et al.,* 2002)	NP_442160.1	TM quality control (Silva *et al.,* 2003)
	slr1604	**FtsH3** (Sokolenko *et al.,* 2002)	NP_440330.1	Indispensable (Mann *et al.*, 2000)
	sll1463	**FtsH4** (Sokolenko *et al.,* 2002)	NP_440797.1	No obvious phenotype (Mann *et al.,* 2000)

***TableVI.1.* Four FtsH proteins present in Synechocystis sp. 6803.** For each FtsH protein both, NCBI accession numbers and the name used by the sequencing centre, Kazusa-cyanoses, (**http://genome.kazusa.or.jp/cyanobase/**) are provided

Looking to identify the orthologs of FtsH2 and *slr*0228FtsH in *T. elongatus* BP1 and *Synechococcus* 7942, a preliminary keyword search (ftsH) was performed. The results of this search in the databases maintained by the sequencing centres of our model organisms, Kazusa DNA Research Institute and Joint Genome Institute (JGI) respectively, not only confirmed the presence of FtsH proteins in both species but also revealed the existence of four *fts*H ORFs in each organism.

It appeared that each of the three examined cyanobacterial species, contained four *fts*H ORFs in their genomes. Nonetheless, as the results of a key-word search are based almost entirely on the adopted by each database nomenclature and most importantly do not provide any information on similarities between the proteins, a homology search was carried out as well.

To perform the homology analyses in the databases the most popular for such purposes sequence similarity search tool BLAST (**B**asic **L**ocal **A**lignment **S**earch **T**ool) was used, while adjusting the algorithm's parameters the BLOSUM 62 (**Blo**cks **Su**bstitution **M**atrix) was the preferred choice of scoring matrix as the most effective in finding all

potential similarities (Baxevanis & Ouellette 2004) and the threshold for Expectation (E) value was set at 0.001.

The results of a series of similarity searches in the databases of the aforementioned centres, having as query the sequence of *slr*0228FtsH protein (627amino acids) confirmed the initial keyword search. Each of our model organisms appeared to contain four distinct *fts*H ORFs of which detailed information as well as on the encoded FtsH proteins is provided in Chapter III, Tables III.2; & III.3. Above all however, the similarity scores in the homology searches (BLAST) revealed the likeliest orthologs to *slr*0228FtsH, in particular 360FtsH in *Synechococcus sp* 7942 and *slr*0734FtsH in *T. elongatus* BP1.

However, because multiple sequence alignment (MSA) is by far more informative than the pair-wise one, i.e. BLAST or FASTA, all twelve FtsH protein sequences were aligned with Clustal W software (Thompson *et al.,* 1994), on the EMBL-EBI database (European Molecular Biology Laboratory – European Bioionformatic Institute; http://www.ebi.ac.uk/Tools/msa/clustalw2/). The default parameters and the Gonnet matrix was used as amino acid substitution score matrix. The results of this alignment were regarded as satisfactory since they matched perfectly the amino acids residues in catalytic domains of proteins in question, as they appear in MEROPS protease database (http://merops.sanger.ac.uk/). The produced similarity scores (percentage identity) between the FtsH proteins in *Synechocystis sp.* 6803; *Synechococcus* 7942 and *T. elongatus BP1* are displayed in *Table VI.2*.

	Synechocystis 6803				*Synechococcus* 7942				*T. elongatus* BP1			
	1	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
***slr*0228 (1)**	100	57	61	45	79	57	58	44	78	57	61	45
***slr*1604 (2)**	57	100	52	50	57	82	51	50	57	81	52	49
***slr*1390 (3)**	61	52	100	46	60	53	56	44	60	52	55	46
***sll*1463 (4)**	45	50	46	100	45	49	42	72	45	50	41	72
360 (1)	79	57	60	45	100	58	58	43	78	57	62	45
703 (2)	57	82	53	49	58	100	53	49	60	52	52	48
417 (3)	58	51	56	42	58	53	100	43	57	81	63	64
745 (4)	44	50	44	72	43	49	43	100	45	50	42	71
***tll*0734 (1)**	78	57	60	45	79	57	59	46	100	60	61	47
***tll*0131 (2)**	57	81	52	50	58	83	54	50	60	100	52	49
***tll*1832 (3)**	61	52	55	41	62	52	63	42	61	52	100	44
***tll*0528 (4)**	45	49	40	72	45	40	64	71	47	49	44	100

Table VI.2. **Similarity scores (% identity) between annotated FtsH proteins in cyanobacterial species of *Synechocystis* 6803; *Synechococcus* 7942 and *T. elongatus*BP-1.**

The similarity scores generated by the multiple sequence alignment (*Table VI.2*), in perfect agreement with the homology search (BLAST) designated 360FtsH and *tll*0734FtsH, as the most probable orthologous proteins to *slr*0228FtsH. Yet more, after careful examination of the scores, it emerged that for each *Synechocystis* 6803 FtsH there is a corresponding protein in *Synechococcus* 7942 and in *T. elongatus* BP1. The observed pattern of relation between the FtsH proteins in cyanobacteria correlates well with the hypothesis of core complement of FtsHases in higher plants, i.e. that for each *fts*H gene or pair of very similar *fts*H genes in one species there is a corresponding gene or pair of *fts*H genes in other species (Yu *et al.,* 2005). Highly consistent with the above proposal is also the phylogenetic analysis of FtsH proteins, in Figure VI.1., and the mutagenesis studies on FtsHases in *Synechocystis* 6803 and *Synechococcus* 7942. Alike the FtsH in *E. coli*, the *slr*1604 & *slr1390* FtsHases in *Synechocystis* 6803 (Mann *et al.,* 2000) and the 703 and 417-FtsHases in *Synechococcus* 7942 (this thesis) all appeared to be of immense importance for cell's viability. Furthermore, *slr*1604FtsH and 703FtsH are most closely related to each other (Table VI.2) and to *E. coli* FtsH (see below) than to any other of the remaining FtsHases. The same pattern of similarities is valid for

*tll0131*FtsH of *T. elongatus* BP1, i.e., high similarity to *slr*1604FtsH & 703FtsH (Table VI.2.), and to bacterial one as well

As far as the other two indispensable FtsHases are concerned, namely *slr*1390FtsH (*Synechocystis* 6803) and 417FtsH (*Synechococcus* 7942), they are believed to have evolved from *slr*0228FtsH and 360FtsH respectively. (for details see below)

Multi sequence alignment are very useful because the sequence analysis and identification of conserved regions and similarities between proteins they produce, can be used to infer important evolutionary relationships represented visually with a phylogenetic tree.

To gain a better insight into the evolutionary relationships between the FtsH proteins in our model organisms, viz., *Synechocystis* 6803; *Synechococcus* 7942; *T.elongatus* BP-1, a phylogenetic tree was constructed, Figure VI.1.

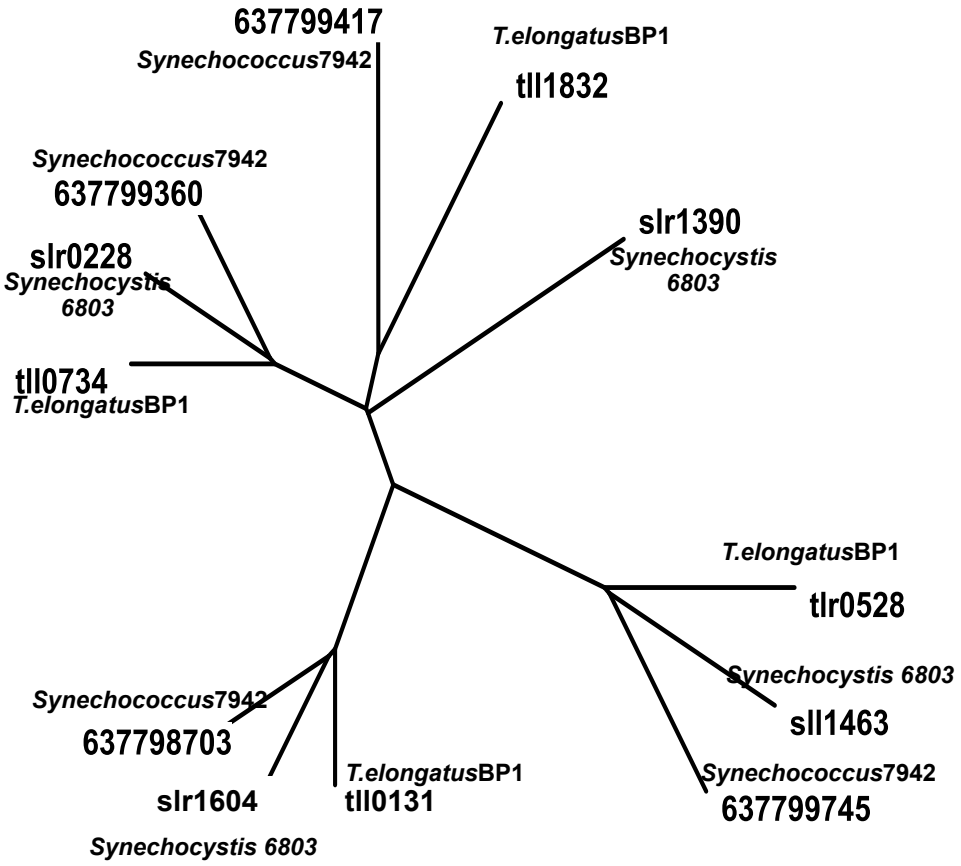

Figure VI.1 **Radial Phylogenetic Tree of putative FtsH proteins from *Synechocystis* 6803, *Synechococcus* 7942 and *Thermosynechococcus elongatus* BP-1.** For the construction of the phylogenetic tree the neighbour-joining distance method (Saitou & Nei, 1987) from EMBL-EBI has been used and the Gonnet matrix was applied to generate the protein distance matrices. Graphical presentation was produced with FigTree Program (http://tree.bio.ed.ac.uk/software/figtree/).

Although unrooted, hence providing no information about ancestry (not the purpose of the present MSA), the phylogenetic tree in Figure VI.1, is very informative and underpins to certain degree the proposal that for each *fts*H gene in one cyanobacterium there is a corresponding gene in an another. This hypothesis correlates reasonably well with that of monophyletic origin of cyanobacteria (Tomitani *et al.,* 2006) although the latter is still an issue under debate.

Regardless however of the origin of cyanobacteria, monophyletic or not, the above phylogenetic tree of cyanobacterial FtsH proteins classifies the twelve FtsHases into four groups, three of which appear as distinct monophyletic clades. It is evident that in each cyanobacterium three of their FtsHases appear to have a corresponding orthologous protein in other species whereas this relationship is not so apparent for the fourth FtsH. Yet the clustering of *slr*0228FtsH, 360FtsH and *tll*0734FtsH into one

monophyletic clade is supported by experimental data, inasmuch as the former two have been shown to be involved in TM quality control mechanism (Silva *et al.,* 2003, Komenda *et al.,* 2006; this thesis).

3. Plant and cyanobacterial FtsH orthologs and their nomenclature

Although life comes in various shapes and sizes, many cellular structures and the functions they perform remain highly conserved, i.e., reaction centers and electron transport reactions. Because such remarkable structural and functional conservation characterizes certain cellular components, it is plausible *ipso facto*, that similar level of conservation will distinguish the elements of their maintenance mechanisms. Proteases are an indispensable part of these regulatory mechanisms maintaining cell's homeostasis under ever changing conditions. Thereby it is reasonable to believe that the proteolytic part of PS-II repair cycle has been phylogenetically conserved between cyanobacteria and green plants. The experimental data thus far correlate highly with this hypothesis disclosing FtsH proteases as a key component of the repair cycle in both plants and cyanobacteria (Bailey *et al.,* 2001; Silva *et al.,* 2003; Komenda *et al.,* 2006). Nonetheless as FtsH proteins exist in several copies in oxygenic phototrophs, mainly four in cyanobacteria and considerably more in green plants (for details see below); as the functional form of the holoenzyme comprises a hetero-oligomeric structure; as our knowledge on the *modus operandi* of the holoenzyme stems largely from studies with *E. coli* and *A. thaliana;* and finally as the number of available genomic DNA sequences increases rapidly while the existent nomenclature depends mainly on the sequencing center, similarity analysis between plant and cyanobacterial FtsHases was performed to identify the orthologous proteins, bring certain coherence to the nomenclature between corresponding proteins and probably to predict the possible structural constituents of cyanobacterial FtsH complex.

Thus, based on the knowledge gleaned from experimental studies and exploiting the availability of plant and cyanobacterial FtsH sequences, a MSA between the twelve FtsH proteins of *Synechococcus* 7942, *Synechocystis* 6803 and *T. elongatus* BP1 and the other twelve of *A. thaliana* was performed. To ensure the best possible results, all sequences before the alignment were grouped by decreasing similarity order, known from similarity searches (BLAST). Yet, before interpreting the similarity scores, the produced alignment was manually checked whether the conserved features of AAA cassette (walker boxes A & B and SRH) and the catalytic domains were correctly

matched. The similarity scores (percentage identity) between the cyanobacterial and plant FtsH proteins are displayed in Table VI.3. It must be said however that exclusively for practical reasons (displaying options), only certain scores are displayed in Table VI3 In particular, the FtsHases from *T. elongatus* BP1 have been omitted while from *A. thaliana* only targeted to chloroplast FtsHases and the FtsH6 are being presented.

	Synechococcus 7942				*Synechocystis* 6803				*Arabidopsis thaliana*						
	360 FtsH	703 FtsH	417 FtsH	745 FtsH	slr 0228	slr 1390	slr 1604	slr 0528	FtsH 1	FtsH 2	FtsH 5	FtsH 6	FtsH 7	FtsH 8	FtsH 9
360FtsH	100	58	58	43	79	60	57	45	50	62	50	57	37	62	36
703FtsH	58	100	53	49	57	53	82	49	61	53	61	49	41	52	40
417FtsH	58	53	100	43	58	56	51	41	45	50	45	47	38	51	37
745FtsH	43	49	43	100	44	44	50	72	46	44	46	43	45	45	44
slr0228	79	57	58	44	100	61	57	45	50	60	50	56	37	60	35
slr1390	60	53	56	44	61	100	52	46	46	50	46	48	37	51	35
slr1604	57	82	51	50	57	52	100	50	61	52	61	48	39	50	38
sll1463	45	49	41	72	45	46	50	100	45	44	46	43	44	44	44
FtsH1	50	61	45	46	50	46	61	45	100	44	86	41	38	44	36
FtsH2	62	53	50	44	60	50	52	44	44	100	44	61	35	89	34
FtsH5	50	61	45	46	50	46	61	46	86	44	100	42	36	45	35
FtsH6	57	49	47	43	56	48	48	43	41	61	42	100	36	63	34
FtsH7	37	41	38	45	37	37	39	44	38	35	36	36	100	36	83
FtsH8	62	52	51	45	60	51	50	44	44	89	45	63	36	100	36
FtsH9	36	40	37	44	35	35	38	44	36	34	35	34	83	36	100

Table VI.3. Similarity scores (% identity) between cyanobacterial and plant FtsH proteins. *In particular: 360FtsH, 703FtsH, 417FtsH, 745FtsH* from *Synechococcus* 7942*; slr0228, slr1390, slr1604, sll1463* from *Synechocystis* 6803*;* and FtsH1, FtsH2, FtsH5, FtsH6, FtsH7, FtsH8, FtsH9 from *A. thaliana.* The FtsHases have been presented in this order (by organism) only for clarity and ease of reference.

Two of *A. thaliana* non-chloroplast FtsHases has drawn some attention, each for different reasons.

The FtsH6 protease, Table VI.3, has been singled out because the *fts*H6 gene remained silent under all examined conditions (Yu *et al.,* 2004). The presence alone of such silent gene in *A. thaliana* inevitably led to comparison with *sll*1463FtsH protein of *Synechocystis* 6803 that has been shown to have no obvious phenotype, at least under the tested conditions (Mann *et al.,* 2000). The similarity scores from the MSA showed no apparent connection between these two proteins. In fact, FtsH6 exhibited noticeably higher similarity to the group of 360FtsHase and it is likely therefore to have evolved from it. For unknown reasons it either remained as a redundant copy or the conditions under which it is expressed have not been found yet.

The other non-chloroplast selected protease was FtsH12, that unlike any other, exhibited the same level of low similarity not only to cyanobacterial but also to its counterparts FtsHases in *A. thaliana*.

As far as the chloroplast encoded FtsHases are concerned, the similarity scores, Table VI.3, show that for almost every cyanobacterial FtsH, exclusive of 745-like ones, there is a corresponding pair of *A. thaliana* FtsHases. It appears thus that the above described hypothesis about the distribution pattern of *fts*H genes, either in cyanobacteria (Figure VI.1), or in plants as proposed by Yu *et al.,* 2005, applies probably to all oxygenic phototrophs. In other words, for each FtsH or a pair of them in one species there is a corresponding one in another organism. The multiplication of FtsHases in cyanobacteria and plants appears to parallel the evolution of oxygenic photosynthesis and the above hypothesis seems to correlates highly with the theory of monophyletic origin of chloroplasts.

While this relationship is plainly evident for the three FtsHases in each cyanobacterium, it is barely distinguishable for the fourth one i.e., 745FtsH; *sll*1463 and *tlr*0582. In *Synechococcus* 7942 and *Synechocystis* 6803, proteases from this group exhibited marginally higher similarity towards the FtsH1 & FtsH5 pair. No such relationship, even so marginal, was observed for *tlr*0528 of *T. elongates* BP-1. With such insignificant similarity, and in the lack of any experimental data to support functional identity between the particular plant and cyanobacterial FtsHases it is difficult to extend this relationship from homology to orthology. Although far from being a conclusive evidence, it is nonetheless possible, especially in the view of functional redundancy of *sll*1463 (Mann *et al.,* 2000), that the absence of distinct similarity to any plant FtsH is

because the evolution of this group of FtsHases in cyanobacteria occurred after the primary endosymbiosis event.

As one of the main purposes for this MSA was to assign the same names to orthologous proteins, and if possible, to predict the structural components of the cyanobacterial FtsH complex, an understanding of the plant FtsH holoenzyme is essential.

Formation of multimeric structure (oligomerisation) appears to be a prerequisite for the proteolytic activity of FtsH proteases (Kihara *et al.,*1996; Shotland *et al.,* 1997; Kihara *et al.,* 1998; Karata *et al.,* 1999). In *E. coli* the active FtsH enzyme exists as a super-complex with HflKC (Kihara *et al.,* 1996, Kihara *et al.,* 1998), in mitochondria it combines with hetero-oligomeric prohibitin complex (Steglich *et al.,* 1999), whereas in plants it forms mainly hetero oligomeric complexes with other forms of FtsH proteins (Sakamoto *et al.,* 2003; Sakamoto 2003).

The FtsH proteins in *Arabidopsis thaliana* are encoded by a multigene family of 16 homologous genes, four of which however are proteolytically inactive (Sokolenko *et al.,* 2002). The twelve active FtsH forms, designated with the numbers 1 to 12 (Adam *et al.,* 2001), occur mostly in pairs of closely related proteins (Yu *et al.,* 2004) three of which (pairs) are located in chloroplast. The FtsH complex in *Arabidopsis thaliana* is composed of Type-A (FtsH1/FtsH5) and Type-B (FtsH2/FtsH8) subunits (Yu *et al.,* 2004; Rodrigues *et al.,* 2011) and the chloroplast biogenesis requires both types, A & B (Sakamoto 2003, Sakamoto *et al*., 2003). Nonetheless, only one isomer from each pair is required for proper functioning of FtsH holoenzyme (Zaltsman et al., 2005). Disruption of *fts*H5 gene produces variegated leaves, phenotype var1, whereas mutation of *fts*H2 results in even stronger variegated phenotype known as var2. Although disruption of the second isomer from each pair, viz., *fts*H1 or *fts*H8 does not affect the leaf pigmentation, over-expression of these two genes can restore var1 and var2 phenotypes respectively to the wild type form (Yu *et al*., 2004; Yu *et al*., 2005). This interchangeability between the proteins in each pair, either Type A or B, but not between the pairs, suggests certain functional redundancy within the pairs. Double mutations in either type of FtsHases are regarded as lethal inasmuch as the mutants can only grow heterotrophically on sucrose medium.

The cyanobacterial FtsHases that are involved in TM quality control mechanism, namely 360FtsH , *slr*0228 and *tll*0734, albeit not experimentally proved for the latter, and the group of 417FtsHases (*slr*1390 and *tll*1832), all invariably exhibited the highest similarity to Type-B chloroplast proteases, viz., FtsH2 and FtsH8.

The group of 703-like proteases (703FtsH, *slr*1604 and *tll*0131), essential for the viability of the cell, showed highest similarity to Type-A chloroplast proteases, viz., FtsH1 and FtsH5. Reversely, when plant FtsHases were compared to cyanobacterial ones, it emerged that the mitochondrial pair, FtsH3 & FtsH10 were closely related to the 703-like proteins. Given the bacterial origin of mitochondria (α-proteobacterial rickettsia), this finding, combined with similarity scores for thousands of bacterial FtsHases (for details see below), suggests that the 703-like proteins constitute the archetypal form of FtsHases in cyanobacteria

While the similarity scores clearly related the 360 & 417-like proteins to Type-B FtsHases, they were nevertheless insufficient to relate them to particular one, i.e., FtsH2 or FtsH8. Similarly, 703FtsHase was not distinctly related to either Type-A FtsHase (FtsH1, FtsH5). For this reason identification of corresponding proteins was based on experimental data. Inasmuch as 360 and 703-like proteins have essential functional roles they are unlikely to be mere redundant copies as FtsH8 and FtsH1 are respectively in *A. thaliana*. Furthermore, as 360-like proteins alike FtsH2 are involved in PS-II repair mechanism, to reduce the interspecies FtsH nomenclature confusion the former will be henceforth referred to as FtsH2, Table VI.4. Similarly, the name FtsH5 will be assigned to 703-like proteins, Table VI.4. As much as we would like to assign the name FtsH8 to 417-like proteins, neither the similarity scores nor the experimental data allow us to do it. Although both 360 and 417-like FtsHases are related to Type-B proteins they are distinct from each other in contrast to FtsH2 & FtsH8 pair that are very closely related.

		Orthologous FtsH	Organism
Type A	**FtsH1**	745FtsH ?	*Synechococcus* 7942
		*sll*1463 ?	*Synechocystis* 6803
		-	*T. elongatus* BP-1
	FtsH5	703FtsH	*Synechococcus* 7942
		*slr*1604	*Synechocystis* 6803
		*tll*0131	*T. elongatus* BP-1
Type B	**FtsH2**	360FtsH	*Synechococcus* 7942
		*slr*0228	*Synechocystis* 6803
		*tll*0734	*T. elongatus* BP-1
	FtsH8	417FtsH ?	*Synechococcus* 7942
		*slr*1390 ?	*Synechocystis* 6803
		*tll*1832 ?	*T. elongatus* BP-1

Table VI.4. Plant and Cyanobacterial FtsH orthologous proteins and their nomenclature.

In vivo studies with *Synechocystis* 6803 and *Synechococcus* 7942 have shown that FtsH2 protease, despite being an essential element of TM quality control mechanism (Silva *et al.,* 2003, Komenda *et al.,* 2006; this thesis), deletion of its gene in either species, did not prevent the formation of functional photosynthetic apparatus nor it affected the normal distribution of TM within the cyanobacterial cells (Chapter V, Figure V.16). In contrast to cyanobacteria, deletion of *fts*H2, or *fts*H5 genes in plants has detrimental effect on TM development, pronouncedly manifested in white sections of variegated leaves (Sakamoto *et al.*, 2003). Moreover, mutation of either one (*fts*H2 or *fts*H5) is not complemented by the presence of the other (Sakamoto 2003). The difference therefore in severity or intensity of *fts*H2 deletion between cyanobacteria and plants shows, if anything, some functional redundancy in case of cyanobacteria, where deletion of *fts*H2 seems to be complemented to an extent by other FtsH or Ftshases. The question then is, which of the remaining three FtsHases compensate(s) for the loss of FtsH2?

Although none of the remaining FtsHases cannot be excluded, the likeliest successor of 360FtsH in building up a functional FtsH complex (especially if the proposed model of

FtsH complex in *A. thaliana* is conserved in cyanobacteria) is 417FtsH. Nevertheless, following insertional mutagenesis strategy it is difficult to show implication of any of the three proteins in the formation of FtsH-complex. This is so because 703 and 417-like proteases appear to be essential for the cell while deletion of 745-like genes does not differentiate, in any apparent way, the mutant phenotype from that of wild type. Probably, instead of deleting, over-expression of either genes or some of them is another way, though indirect, to find answer to this question. If in an FtsH2-less mutant, over-expression of either genes or some of them restores the mutant's phenotype closer to the wild type, then it can be deduced that the particular FtsH(ases) is (are) actively involved in the formation of a functional FtsH-complex. Furthermore, over-expression is not only the way to identify the potential subunit(s) within the complex but also, by logical deduction, to elucidate whether the absence of FtsH2 affects the development of the complex, or its performance. Although we know that the deletion of *fts*H2 certainly affects the quality state of photosynthetic apparatus (Mann *et al.,* 2000) and reduces the capacity of the repair cycle (Silva *et al.,* 2003, Komenda *et al.,* 2006, this thesis) we still do not know how this happens. In particular, it is unclear whether the reduced ability of the repair mechanism to maintain a fully functional photosynthetic apparatus is due to the reduced number of fully operational FtsH-complexes or it is due to altered composition (replaced FtsH2) and therefore reduced operational capacity of the FtsH holoenzyme.

In *A. thaliana* for instance, it has been shown that deletion of either *fts*H2 or *fts*H5 genes prevents, the accumulation of the other, suggesting thus coordinated regulation at protein level (Sakamoto 2003). Although we do not know how the mutation of *fts*H2 in cyanobacteria affects the whole complex, we favor the second one, i.e., reduced accumulation of functional holoenzymes due to coordinated accumulation of FtsHases. We are in support of this, because at first place it is difficult to comprehend how such a large enzyme with altered composition and stoichiometry will provide the proper positioning and orientation to the amino acid residues of the catalytic site to perform specificity and precision requiring catalysis as for instance targeting the damaged photosystems only, or degrading exclusively the inactivated D1 subunits and so on. In favor of this hypothesis are also the results of MSA revealing that cyanobacteria alike higher plants, contain two types of FtsHases, A & B, Table VI.3. While 703FtsH exhibit higher similarity to Type-A (FtsH1 and FtsH5), the 360FtsH and the other essential protease, 417FtsH, are closely related to Type-B (FtsH2 and FtsH8). With two Type-B proteins, 360FtsH and 417FtsH and at least one Type-A protein, 703FtsH, (similarity

scores of 745FtsH are not as distinct), it appears that the compositional model of FtsH holoenzyme is at its core conserved in oxygenic phototrophs.

The difference, if any, seems to exist at the level of coordinated accumulation of the two types of FtsHases. In plants simply this interaction looks considerably stronger. Absence of one type subunit prevents the formation of functional complexes by the proteins of the other type, which are likely to be rapidly degraded. It has been reported that multisubunit complexes involved in photosynthesis are broken down when a constituent protein is missing (Wolman *et al.,* 1999; Hippler *et al.,* 2002).

Nonetheless the presence of two types of FtsH-proteins as a prerequisite for the accumulation of FtsH-complex may not be exclusive of cyanobacteria. The 703-like proteins as the ancestral form of FtsHases in cyanobacteria might comprise the basis for formation of functional complexes and the lethality of *703fts*H deletion may be due to inability of the cell to form any operational FtsH-complex.

The described above high dependency between the two types of FtsHases for the formation of functional complexes in plants, is not always as strong however. In green sections, fully functional FtsH-complexes are formed despite the fact, that the cells have mutant genotype (FsH2 or FtsH5-less).

After all, the differences in principles ruling the development of a fully functional FtsH-complex in cyanobacteria and plants may ultimately be considerably smaller. In fact, the model of FtsH-complex ontogenesis as a whole, looks like being evolutionary conserved in all oxygenic phototrophs. Any of the observed irregularities comprise ultimately a natural, inescapable concomitant of evolution, not only of a single-celled organism to a multicellular one but also of a cyanobacterium endosymbiont to a chloroplast. Probably the best way to elucidate these important aspects of FtsH-complex formation, considering the significance of 703 and 417FtsHases for the cyanobacterial cell, is to create double mutants that lack either 703 or 417*fts*H genes but at the same time overexpress 360 and / or 745*fts*H

4. Studying the distribution of FtsH among living organisms.

Technological advancements combined with the development of numerous methods for DNA sequencing and the eruption of computational sciences led the cataloging and mapping of the hereditary material to an unprecedented level. Literally thousands of species, strains, viruses, viroids, even environmental DNA samples (metagenomes) distinguished by billions of nucleotide sequence data have been sequenced in recents

years and the number of ongoing projects is still rising. Availability of such data almost dictated the need to study these proteases further.

Although FtsH proteins have been reported to exist in all living organisms apart from Archaea (Tomoyasu *et al.,* 1993; Swaffield & Puruggan 1997; Langer, 2000) the richness and diversity of the presently available data is such that allows not only to put that thesis to the test but to try find answers to a series of other, intriguing questions, i.e., do all cyanobacterial FtsHases have their orthologs in eukaryotes; which FtsH is the ancestral form; what is the evolution pattern of cyanobacterial FtsHases; which subunits comprise the cyanobacterial FtsH holoenzyme and many others.

The fast-paced submission of new sequences into the Databases can obviously set the results of a research outdated soon after the study. Although correct when seen from a certain angle, this standpoint does not necessarily affect the correctness or the credibility of a research, yet more when considering the essence and the type of conclusions and not to forget the size of the samples they have been inferred from. Inasmuch as conservation between protein's function and structure underlies the basic principle of proteins evolution, accumulation of new data in the databases is likely to bring more clarity rather than to change the essence of our understanding on the pattern of FtsH distribution, function and evolution. In other words, addition of more details into the current, impressionist-like picture, instead of changing the main theme can transform it into an elaborate one of photographic accuracy. Thus, the conception for instance that bacteria contain a single *fts*H gene, remains largely correct despite the recent knowledge than many species, including some *E. coli* strains, contain two or more *fts*H genes (for more details see below).

In search for distribution of FtsH proteins in cellular organisms the NCBI Similarity search tool, protein-protein BLAST suite, was used and the search was performed on NCBI Reference Sequence (refseq_protein) Database. As cited on NCBI web page the RefSeq Database is 'A comprehensive, integrated, non-redundant, well-annotated set of reference sequences including genomic, transcript, and protein'. Unless otherwise stated, as query in these homology searches, the whole length FtsH sequences of *Synechococcus* 7942 and often those of *Synechocystis* 6803 or other bacterial species were used.

Looking to identify the distribution patterns of FtsHases in various groups of cellular organisms a multitude of BLAST searches against the Reference Sequence database

were performed. These similarity searches against the entire database although very useful to study the presence of homologs in groups of organisms of higher taxonomic hierarchy i.e., kingdoms and phyla, are less informative for lower taxonomic ranks such as classes, families and genera. For that reason another series of homology searches were performed against all bacterial and eukaryotic phyla, containing photosynthetic organisms. As a result, a number of new hits and organisms, not present in the overall searches were identified. Nonetheless as even the BLAST searches against the selected group of organisms are not guaranteed to identify all FtsHases present in one organism, to confirm therefore unambiguously the number of these proteins in each species, endless homology searches against each organism individually were performed as well. As a result of this adopted strategy it emerged that several species, e.g. *Paulinella chromatophora,* contained more FtsHases than initially identified. Furthermore to exclude the possibility of missing hits due to incomplete Genomic DNA sequencing, especially when considering that many photosynthetic eukaryotes have four DNA sources to store information i.e., nucleous, mitochondrial, plastid and nucleomorph DNA, the sequencing status for each organism was also checked individually.

All reports in this study are based exclusively on NCBI taxonomy, hence the NCBI taxonomy ID (taxid) for every group or species are provided, for both consistency and ease of reference. Yet, when looking for FtsH distribution in phyla containing photosynthetic organisms, as such were considered any organism capable to one or another degree or at any stage of its life (facultatively, obligately, mixotrophically etc) to perform chlorophyll-based photosynthesis.

4.1. Distribution of FtsH in cellular organisms

The homology searches in Reference Sequence database produced some interesting results, Table VI.5. BLAST search with FtsH5 (703FtsH) as query generated characteristically the larger number of hits than the rest of FtsHases. The protein that seemed to be involved in TM quality control mechanism of *Synechococcus* 7942, i.e., FtsH2 (360FtsH), had the second largest number of hits and considerably larger than those produced by 417FtsH and 745FtsH that generated very similar results, Table VI.5.

The observed discrepancy in the number of hits generated by each protease is an indication that the *fts*H genes in cyanobacteria, although unlikely to have derived from lateral gene transfer, have diverged considerably from each other, and some are not exclusive to have derived from a secondary duplication event.

The substantially higher number of hits and organisms the homology search with FtsH5 has produced, and given that this difference comes from the domain of bacteria, is but a strong indication that this particular protease might be the ancestral FtsH in cyanobacteria. The hypothesis is consistent with similar research, e.g. Chen *et al.,* 2000.

Nonetheless, if FtsH5 is to be the archetypal form, then there seems to be a certain inconsistency in the results, at least as far as the number of hits in eukaryotes is concerned, Table VI.5. This seeming discrepancy between the expected and the produced hits in the domain of eukaryotes, may ultimately not be as contradictory when considering the type of organisms the difference has been identified in, and the *modus operandi* of FtsH-complex. The results, nearly identical in all eukaryotic phyla (data not shown), differ significantly in the kingdom of Green Plants (*Viridiplantae*), and most particularly in vascular plants. In these organisms as mentioned earlier on, the FtsHases come in pairs of closely related proteins, and the FtsH-complex is composed of two types of FtsHases, A & B. The MSA between cyanobacterial and plant FtsHases has shown that while Type-B proteins in cyanobacteria is represented by two FtsHases (360 and 417), Type-A has only one, i.e., 703FtsH. It is plausible therefore, that the observed difference in the number of hits stems from the fact that the chloroplast has inherited two Type-B proteins and only one Type-A.

	Number of hits - Number of organisms			
	360FtsH	**703FtsH**	**417FtsH**	**745FtsH**
Cellular organisms [taxid: 131567]	1773 - 1503	2327 - 2106	1301 - 982	1320 - 1083
Bacteria [taxid: 2]	1683 - 1460	2278 - 2078	1211 - 940	1318 - 1082
Eukaryotes [taxid: 2759] Viridiplantae [33090]	90 - 43 65-20	49 - 28 36-27	90 - 42 64-20	2 - 1 0-0
Archaea [taxid:2157]	~~1155~~ - 230	~~1157~~ - 230	~~1153~~ - 230	~~1150~~ - 230

Table VI.5. **Taxonomy reports on homology search (BLAST) having as query the FtsH proteins of *Synechococcus* 7942.** For consistency and ease of reference the NCBI taxonomy IDs, i.e., taxid, are provided for each group of organisms. The typographical representation of numbers with a strikethrough, illustrating the hits in archaea, is because almost none of them in actuality corresponds to a real FtsH. These numbers are the result of separate BLAST searches against the domain of Archaea since the homology searches in cellular organisms produced no hits in this domain. Nearly all hits correspond to AAA proteins, therefore, by definition of FtsH proteins, certain degree of similarity is inevitable.

Another distinguishing characteristic of these similarity searches in cellular organisms is the number of hits produced by 745FtsH. Unexpectedly, this BLAST showed no hits among eukaryotes apart from two, and these in just one organism, the freshwater ameboid *Paulinella chromatophora* from the phylum of *Rhizaria*. Identical results to these were produced in the search with the sequence of *sll*1463 as query. The fact that this homology search generated hits in one single organism may suggest that the evolution of 745-like FtsHases in cyanobacteria has occurred after the primary endosymbiosis event with a eukaryotic cell. Nevertheless, as the particular protease seems to be a redundant copy or auxiliary, expressed under specific conditions, the possibility that this gene in the symbiont was either deleted or has changed greatly evolving into a paralog, cannot be ruled out. With none of the aforementioned possibilities being mutually exclusive, there are certain other facts, including some about this remarkable organism, *Paulinella chromatophora,* that merit more attention before drawing any conclusions.

Paulinella chromatophora is the only known species in this genus that through endosymbiosis with a cyanobacterium, believed to be related to *Synechococcus* or *Prochlorococcus* (Marin *et al.,* 2005), is capable of photosynthesis. In that sense it contrasts even with its close relative *Paulinella ovalis,* that contains no plastid and remains to date an active predator of cyanobacteria (Johnson *et al.,* 2005)

The chloroplast (as a semi-autonomous, photosynthesizing nascent organelle) in this organism differs significantly not only from that of green plants but also from the rest of photosynthetic organisms of the same phylum, *Rhizaria*.

The uniqueness of *P. chromatophora* chloroplast, in comparison to that of Green Plants lies not in the origin but in the evolutional time. Thus, notwithstanding that chloroplasts of both, *Viridiplantae* and *P. chromatophora*, descend directly from a cyanobacterial cell, the latter, with evolutionary history of only 60 million years (Marin *et al.,* 2005; Yoon *et al.,* 2006; Nowack *et al.,* 2008), might be regarded as an infant when compared to 1.5 billion years of evolution of the former (Blair *et al.,* 2005; Yoon *et al.,* 2004). Yet, the chloroplast of *P. chromatophora* differs from that of other photosynthetic amoeboids *Rhizaria* in the origin, since in the latter it has evolved from secondary endosymbiosis with a green alga. The chloroplast of *P. chromatophora* therefore comprises not only evidence for phagotrophic origin of plastids, but also an excellent case to study the evolution of various cyanobacterium-derived proteins as well as theirs genes lateral transfer from the endosymbiont genome to the host nucleus.

Homology search against the genome of *P. chromatophora* in particular, revealed the presence of three FtsHases, Table VI.6. When the Maximum Scores between the FtsHases of *Synechococcus* 7942 and those of *P. chromatophora* were compared, surprisingly one FtsH in the amoeboid (NCBI Accession: YP_002048793.1) exhibited markedly the highest similarity towards 745FtsH.

Cyanobacteria in principle appear to contain four distinct forms of FtsHases, nevertheless, there are many other species, e.g. *Anabaena sp*. 90 (taxid 46234); *Cyanobacterium aponinum* PCC 10605 (taxid 755178); *Synechococcus* ps. JA-2-3B'a(2-13) (taxid 321332); with three or even two FtsHases, Table VI.6.

Contrasting the primary origin of chloroplasts in Green plants, Brown and Red algae, the lateral transfer of *fts*H genes from the endosymbiont genome to the host nucleus, was not as universal, inasmuch as Red & Brown algae contain a single *fts*H gene in their plastid genome (Reith 1995).

Taking into consideration all of the aforementioned facts, as well as the similarity scores that the 745-like FtsHases exhibit towards chloroplast and mitochondrial FtsHases in *A. thalina,* (Table VI.3) and all eukaryotic homologs, Table VI.6 to that of *Paulinella chromatophora*, it seems that the 745FtsH has evolved in cyanobacteria after the primary endosymbiosis. In other words, the 745FtsH seems to contain no true orthologs amongst eukaryotic photosynthetic organisms, excluding of course species such as *P. chromatophora.*

4.2. Do the FtsH proteins exist in Archaea?

All homology searches against cellular organisms, invariably showed no hits in the domain of Archaea. And though these results were consistent with several articles reporting the absence of FtsH proteins from this domain, e.g., Tomoyasu *et al.,* 1993; Swaffield & Puruggan 1997; Langer, 2000, the richness and diversity of presently available genomic data, along with certain dose of the omnipresent curiosity, led to a series of BLAST searches against Archaea and some individual organisms.

Regardless of the origin of the query FtsH sequence, either bacterial or cyanobacterial, characteristically, all homology searches in this domain generated nearly the same number of hits distributed within the same number of species, Table VI.5. Although practically none of the hits belonged to FtsH family, the presence of one or two AAA cassettes typified the hits in principle. Proteins with two AAA modules usually exhibited slightly higher similarity to the query FtsH compared to those with one AAA

module. Furthermore, when the similarity scores of all FtsHases were compared, it appeared that the hits were predominantly related to either bacterial or 703FtsH.

Notwithstanding that virtually none of the hits belong to FtsH proteins, the fact that those with two AAA cassettes were referred to as "cell division proteins", a name commonly ascribed to FtsHases has to be stressed, much more when considering their universal distribution among all living organisms.

It is known that in many bacteria there are several proteins required for cell division, though for most of them the exact functions are not well understood. In *E.coli* for instance, the products of more than nine essential genes (*fts*A, *fts*I, *fts*K, *fts*L, *fts*N, *fts*Q, *fts*Z, etc) are required for proper septation (Lutkenhaus & Addinall. 1997). And though not all of these proteins belong to AAA family, some as FtsZ are GTP dependent, they are all members of the Walker-type superfamily.

If archaea, bacteria and eukaryotes constitute separate lines of descent diverging from an ancestral form of life which as described in Woese 1998, ' is not a discrete entity, but rather a diverse community of cells that survives and evolves as a biological unit', then the universal spread of AAA-type proteases suggests their existence in these ancestral 'cells' defined as progenotes (Woese & Fox 1977). And though progenotes are neither organisms or cells in any conventional sense of the words, they nonetheless possessed a rudimentary mechanism for genome replication, gene expression and cell division. Thus, AAA-type proteases must had been an indispensable part of progenotes cell-division machinery.

Careful examination of more than thousands hits in 230 organisms (Table VI.4.), revealed that with the exception of just two organisms, none of the remaining archaeans contained an FtsH homolog. In particular, two proteins (YP_004071473.1 & YP_004071311.1) in *Thermococcus barophilus* MP (taxid: 391623) and three (YP_002582739.1; YP_002582772.1; YP_002583353.1) in *Thermococcus sp*. AM4 (taxid 246969) were designated as "cell division FtsH proteins".

Identification of FtsH proteins amongst Archaea was an unexpected fact and therefore included an element of surprise. Beside that however, closer examination of the results raised several questions, about the absence / rarity of FtsHases in Archaea and especially about the chosen nomenclature for the five particular proteins.

The rare occurrence of FtsHases in Archaea (in just two out of 230 organisms) along with their absence form closely related species from the same genus, although

surprising, is a phenomenon that do occur in nature and it is often explainable. Thus for instance while *Paulinella chromatophora* is an active phototroph because of cyanobacterium-derived chloroplast, its close relative *Paulinella ovalis* is a heterotroph and remains to date an active predator of cyanobacteria. More characteristic is the case of some alpha-proteobacteria from the genus *Bradyrhizobium* that through their symbiotic relationship with plants, fix atmospheric nitrogen. Interestingly, while many strains retain the capability for heterotrophic photosynthesis, others have lost it altogether (Wong *et al.,* 1994; Molouba *et al.,* 1999). In like manner, the two *Thermococcus* organisms are plausible to have acquired *fts*H genes through lateral gene transfer, or alternatively retained these genes under particular selective pressure, in contrast to other archaean.

FtsH is one of the most important regulatory proteins in membrane biogenesis because it controls the balanced synthesis of phospholipids and lipopolysaccharides, and yet the quality of membrane proteins by both assisting their assembly and degrading any abnormal forms (Ogura *et al.,* 1991; Ogura *et al.,* 1999). The significance of FtsH however in bacterial world varies depending on cell's structural organization, viz., two membranes (inner & outer) in Gram-negative or one just one in Gram-positive. As a result the lethality of FtsH mutations in Gram-negative bacteria, *E. coli,* where lack of this protease causes unbalanced production of outer membrane structural components (LPS, phospholipids), the dispensability of the particular protein in Gram-positive, *Bacillus subtilis (*Deuerling *et al.,* 1997*),* seems to be reasonable inasmuch as they do not possess outer membrane.

Within the world of prokaryotes, the cell structure and membrane composition of archaea differs strongly from that of bacteria (Koga & Morii 2007). With just one membrane, archaea resemble more the Gram-positive bacteria (Gupta 1998). In contrast however to glycerol-ester lipids of bacterial and eukaryotic membranes, the archaean's are made of glycerol-ether lipids (De Rosa M *et al*., 1986). Most striking difference of all is that the lipid-bilayers of the former, is often replaced by a monolayer in the latter. Yet, the cell wall, present in most Archaea (Golyshina *et al*., 2000) differs profoundly from the bacterial one, because instead of peptidoglycan it contains surface-layers proteins (Sára & Sleytr 2000).

Considering thus the weight of FtsHases in the world of monoderm and diderm bacteria along with the series of profound differences in membrane organization and composition between archaea and bacteria, it may be then this diminished necessity for

FtsHases to be the key factor explaining their absence from this domain. Under these particular selective conditions the FtsH proteins either have not evolved in this domain or have been deleted if they were ever inherited from the progenotes.

As mentioned earlier on, analysis of five particular FtsHases raised questions about the chosen nomenclature of these proteins. These proteins were referred to as FtsHases albeit most of the hits, including those in other *Thermococcus* species, exhibited the same similarity. Furthermore, four of the FtsHases in question appeared to contain two AAA-modules contrary to one that FtsH-type proteins by definition are supposed to contain. And though clear-cut classifications of any sort, being that organismal or molecular, are most often than not exceptions than rules, designation of these proteins as FtsH, despite such a major divergence from definition combined with low overall similarity (Maximum score) is not well understood. Another objection to the chosen nomenclature is the absence from the NCBI feature list of any region or Conserved Domain (CD) characterizing or distinguishing FtsH proteins, e.g., Peptidase M41 (ID: pfam01434) or FtsH family (ID: TIGR01241)

In conclusion, if the proteins in question, despite the stressed objections, fulfill the structural and functional criteria for FtsH proteases, then their presence in two *Thermococcus* organisms only, can be explained by two different conventions, given the lack of evidence for their existence in progenotes. First, deletion of these proteins from the vast majority of organism in this domain due to diminished use or necessity or alternatively acquisition of *fts*H genes by the two organisms by the means of lateral gene transfer or convergent evolution.

4.3. Distribution of FtsH amongst bacteria

FtsH-type proteins are ubiquitous in the world of bacteria, and in general are believed to exist as a single copy. Nonetheless their distribution is not always uniform as there are species, even entire groups or phyla where they are present in greater numbers. The study of such cases is helpful to acquire a comprehensive picture of their distribution and yet may also help to understand some of the reasons for their multiplication. It has been proposed for instance that the evolution of FtsHases into multigene families in cyanobacteria correlates with the evolution of oxygenic photosynthesis, a relationship manifested more emphatically in higher plants.

To gain a better understanding of FtsH distribution, find out whether similar relationships have evolved in other photosynthetic bacteria, and to identify the ancestral

FtsH form in cyanobacteria, multitude of homology searches were performed against several phyla. As the subject of this project is in the great field of photosynthesis, and bacteria on the other hand side comprise the largest and most diverse domain of life, inevitably this search was mainly confined to the six phyla containing photosynthetic organisms. However, searching for the ancestral form of FtsH in cyanobacteria, BLAST search in other two ancestral phyla with no photosynthetic organisms viz., Actinobacteria and Deinococcus-Thermos (Gupta 2005) was also performed.

Chlorophyl-based photosynthesis was known until recently to occur in only five phyla. With the discovery of a photosynthetic acidobacterium, i.e., *Chloracidobacterium thermophyllum,* in 2007, photosynthetic organisms are now distributed in six phyla, namely: Firmicutes (Heliobacteria); Chloroflexi (green non-sulphur bacteria); Chlorobi (green sulphur bacteria); Proteoabacteria (Purple bacteria); Cyanobacteria; and Acidobacteria.

Firmicutes

In general, the Gram-positive Firmicutes appeared to contain a single copy of FtsH, including the photosynthetic species of *Heliobacterium modesticaldum Ice1,* Table VI.5. Nonetheless, species with two or even three of these proteases were also identified. If *Firmicutes*, as suggested stand at the base of bacterial evolutional tree, then the presence of several FtsH copies is an indication that the multiplication of these proteases is an early event, not confined just to cyanobacteria. Homology search in *Heliobacteriaceae* (taxid: 31984), the only family in the phylum known to have photosynthetic organisms, produced just a single true hit in the aforementioned species. Absence of hits in other photosynthetic organisms from this family was solely due to incomplete genome sequences of other species. Almost invariably, all hits in this phylum exhibited the highest similarity to FtsH5 (703FtsH), while in species with more than two FtsHases, some of the hits were closely related to 745FtsH.

Chloroflexi

Search for homologous FtsH proteins in the deep branching phylum of *Chloroflexi* showed that these organisms in principle contain a single FtsH albeit exceptions with three of even five FtsHases are also present, Table VI.5. As far as the photosynthetic species are concerned, only one out of six, i.e., *Oscillochloris trichoides* DG-6 appeared to contain two FtsHases, Table VI.5. Once again, all identified FtsH proteins, invariably exhibited the highest similarity to FtsH5. Understanding the reasons for multiplication of *fts*H genes, up to five copies in some species, is difficult. Despite containing no outer

membrane, these organisms stain Gram-negative. Besides, there is weak phylogenetic evidence to support the monophyletic origin of the six classes comprising the phylum (Gupta *et al.,* 2012).

Chlorobi

In some contrast to *Firmicutes* and *Chloroflexi,* the obligate anaerobic photoautotrophic *Chlorobi* (Green Sulphur Bacteria) consistently appeared to contain two FtsHases, Table VI.5. One of two hits in each species, alike most of bacterial FtsHases exhibited higher similarity to FtsH5, while the second one, quite unexpectedly, was closely related to 745FtsH.

Acidobacteria

Investigation for the distribution of FtsHases within the ubiquitous in soils but physiologically diverse *Acidobacteria* (Quaiser *et al.,* 2003) have shown that all members of this phylum, with just one exception, appeared to contain a single FtsH copy, Table VI.5. For some reasons, the only photosynthetic species, *Candidatus Chloracidobacterium thermophilum,* has two distinct form of the protease. Nevertheless, the size of the sample in *Chlorobi*, mere eight organisms, is rather to small to allow any association of FtsH multiplication with photosynthesis.

Proteobacteria

Proteobacteria comprise a major phylum characterized by eminent phenotypic diversity. Taxonomically divided into six classes referred to by the greek letters alpha through zeta, the group, distinguished by metabolically versatile lifestyles ranging from chemolithotrophs to photosynthesizers, also includes a number of notorious pathogens such as *Salmonela, Escherichia, Yersinia* (plague), and the causative agent of cholera - *Vibrio* (Gupta 2000; Madigan & Martinko 2005). Homology searches were performed only among Purple photosynthetic bacteria, viz., alpha & beta-proteobacteria containing Purple Non-Sulphur Bacteria, and gamma-proteobacteria with Purple Sulphur Bacteria.

α-proteobacteria

Most photosynthetic species were identified among alpha-proteobacteria, fifty-two in particular, and nearly all of them appeared to contain a single copy of FtsH proteins, Table VI.5. Several organisms among them, most noticeably the nitrogen fixing bacilli from genus *Bradyrhizobium,* seemed to have two or even more of these proteases. The distribution of FtsHases on the other hand side among non-photosynthetic alpha-proteobacteria, 390 species, followed the same pattern observed in photosynthetic

representatives, i.e. one FtsH per organism with some exceptions containing up to four copies, Table VI.5.

β-proteobacteria

The majority of 350 beta-proteobacteria were characterized by the presence of a single FtsH, albeit far too many species to be called exceptions appeared to contain two copies, Table VI.5. Only three photosynthetic species were identified among the hits and two of them, typically for this class contained just a single FtsH, Table VI.5.

γ-proteobacteria

Presence of one FtsH in each organism typified the homology searches among gamma-proteobacteria. And though species with two FtsH copies were also identified, the discovery of *E.coli* strains amongst the exceptions was most surprising. As far as the purple sulphur bacteria are concerned, just nine out of 1500 species were identified as such. However, in contrast to non-photosynthetic, most of the phototrophic gamma-proteobacteria were characterized by the presence of two or more FtsHases, Table VI.5.

Actinobacteria & *Deinococcus-Thermus*

In search for the archetypal form of FtsH, two additional phyla were also examined. Both, *Deinococcus-Thermus* and *Actinobacteria* contain no photosynthetic organisms and are believed to consist some of the earliest branching phyla of the eubacterial phylogenetic tree.

BLAST against the former generated relatively small number of hits, less than hundred in nearly twenty species. Predominantly, most of the species appeared with two FtsHases, even though species with either one or three of these metalloproteases were also identified.

Homology search against *Actinobacteria* produced on average nearly 1200 hits distributed in nearly 500 different species and strains. In contrast to *Deinococcus-Thermus* most of the *Actinobacteria* contained a single FtsH although many appeared with two and seldom with three distinct forms of these proteases, Table VI. 5.

Looking for the ancestral cyanobacterial FtsH, the similarity scores to FtsH5; FtsH2; 417, and 745FtsHases from thousands of hits were compared. Unexceptionably, the hits from single-FtsH-containing species, in all eight examined phyla, exhibited noticeably highest similarity towards FtsH5, followed by FtsH2. The results however for species with more than two FtsHases were not as unambiguous and differ among phyla and

classes. The difference in these cases was not in what is the highest similarity towards but how the rest of the hits are related to the four cyanobacterial FtsHases. Thus, again and without any exception, the higher similarity was towards the FtsH5 followed by FtsH2. However, in many *Firmicutes,* alpha & beta-proteobacteria, in *Candidatus Chloracidobacterium thermophilum* and in all Green sulphur bacteria, there is usually one hit that exhibit the highest similarity towards the type 745FtsH. As this group of proteases seems to have, if any, auxiliary functions in cyanobacteria, and no orthologs in *Arabidopsis thaliana* that could allow us to draw some inferences about their function, then this similarity towards one of the FtsHases in green sulphur bacteria, or in other groups of bacteria mentioned above, might be proved useful to provide, even obliquely, important information about their potential role.

As far as the archetypal FtsH in cyanobacteria is concerned, all data collectively lead to a single conclusion, that FtsH5 is the ancestral form whereas FtsH2 seems to be its first derivative.

Table VI.6. **Similarity scores of bacterial to *Synechococcus sp.* 7942 FtsHases.** The similarity scores represent the Maximum scores (the highest alignment score for a set of sequences, calculated from the sum of the match points and the mismatch, gap open penalties). The sorting order produced by Max. Score is usually the same as that produced according to E-value. Underlined species names correspond to non-photosynthetic species. Underlined names. Only elective samples for non-photosynthetic species are provided for each phylum to serve as reference point to the main text.

Bacterial Species	FtsH NCBI Accession	Similarity scores			
		360FtsH	703FtsH	417FtsH	745FtsH
Firmicutes [taxid: 1239]					
Heliobacterium modesticaldum Ice1	YP_001679885.1	546	625	465	500
Mitsuokella multacida DSM 20544	ZP_05404656.1 ZP_05405046.1	566 455	637 481	483 427	519 506
Selenomonas sputigena ATCC 35185	YP_004413822.1 ZP_05898235.1	556 556	641 640	475 475	516 516
Carboxydothermus hydrogenoformans Z-2901	YP_359086.1	561	623	486	506
Thermincola potens JR	YP_003638994.1	561	630	473	500
Clostridium beijerinckii NCIMB 8052	YP_001307246.1 YP_001311846.1 YP_001310132.1	504 471 481	566 494 491	442 414 420	473 509 509
Eubacterium rectale ATCC 33656	YP_002936460.1 YP_002939339.1 YP_002936612.1	469 452 471	518 501 476	434 446 425	420 513 486
***Chloroflexi* - Green Non Sulfur Bacteria** [taxid: 200795]					
Chlorflexus aggregans DSM 9485	YP_002463968.1	545	601	541	466
Chloroflexus auranticus J-10-f1	YP_001635641.1	549	603	540	470
Chloroflexus sp. Y-400-f1	YP_002569923.1	549	603	540	470
Oscillochloris trichoides DG-6	ZP_07684983.1 ZP_07685291.1	529 478	605 539	540 499	464 488
Roseiflexus castenholsii DSM 13491	YP_001430856.1	555	602	530	472
Roseiflexus sp. RS-1	YP_001278646.1	554	601	527	473
Caldilinea aerophila DSM 14535 = NBRC 104270	YP_005443526.1 YP_005440218.1 YP_005440885.1	537 516 508	583 567 533	553 534 509	474 499 468

Bacterial Species	FtsH NCBI Accession	Similarity scores			
		360FtsH	703FtsH	417FtsH	745FtsH
Ktedonobacter racemifer DSM 44963	ZP_06966418.1	532	593	545	493
	ZP_06967442.1	490	534	478	469
	ZP_06970026.1	485	532	488	465
	ZP_06966340.1	473	498	442	468
	ZP_06967958.1	396	426	407	381
Chlorobi - Green Sulfur Bacteria [taxid: 68336]					
Chlorobaculum parvum NCIB 8327	YP_001999330.1	496	527	447	466
	YP_001997701.1	468	503	413	532
Chlorobium chlorochromatii CaD3	YP_378707.1	494	515	437	457
	YP_378329.1	463	495	423	526
Chlorobium ferrooxidans DSM 13031	ZP_01386079.1	502	540	447	459
	ZP_01386541.1	462	502	421	536
Chlorobium limicola DSM 245	YP_001942430.1	502	524	458	464
	YP_001942172.1	463	495	408	524
Chlorobium luteolum DSM 273	YP_375619.1	498	518	442	459
	YP_374011.1	465	504	411	530
Chlorobium phaeobacteroides BS1	YP_001960382.1	507	522	443	454
	YP_001129665.1	466	496	408	537
Chlorobium phaeovibrioides DSM 265	YP_001131017.1	491	524	433	456
	YP_001129665.1	476	500	422	536
Chlorobium phaeobacteroides DSM 266	YP_910908.1	508	545	452	466
	YP_913086.1	459	491	413	531
Chlorobium tepidum TLS	NP_661201.1	489	525	442	460
	NP_661033.1	465	508	413	536
Chloroherpeton thalassium ATCC 35110	YP_001996372.1	505	553	454	471
	YP_001996274.1	466	531	429	547
Prosthecochloris aestuarii DSM 271	YP_002016444.1	494	520	458	453
	YP_002014830.1	472	498	415	529
Pelodictyon phaeoclathratiforme BU-1	YP_002017406.1	498	533	435	448
	YP_002017054.1	484	518	425	521
Acidobacteria [taxid:131550]					
Candidatus Chloracidobacterium thermophilum	YP_004862851.1	520	553	449	479
	YP_004863476.1	451	495	409	532
Acidobacterium capsulatum ATCC 51196	YP_002755094.1	530	569	461	490
Holophaga foetida DSM 6591	ZP_09577544.1	506	538	432	487
Alphaproteobacteria - Purple Non Sulfur Bacteria [taxid: 28211]					
Ahrensia sp. R2A130	ZP_07375277.1	488	559	437	459
Bradyrhizobium japonicum USDA 110	NP_773786.1	504	572	451	477
Bradyrhizobium japonicum USDA 6	YP_005607136.1	506	570	451	476

Bacterial Species	FtsH NCBI Accession	Similarity scores			
		360FtsH	703FtsH	417FtsH	745FtsH
Bradyrhizobium sp. BTAi1	YP_001242667.1 YP_001241240.1 YP_001243535.1 YP_001237948.1	501 472 460 427	555 502 502 486	449 414 399 382	467 516 537 531
Bradyrhizobium sp. CCGE-LA001	ZP_16041857.1	506	570	451	477
Bradyrhizobium sp. ORS 278	YP_001203324.1 YP_001204872.1 YP_001207818.1	502 474 423	556 503 482	450 413 382	466 516 524
Bradyrhizobium sp. ORS 285	ZP_09474415.1 ZP_09472185.1 ZP_09473817.1	504 475 425	556 508 488	448 416 385	468 516 535
Bradyrhizobium sp. ORS 375	ZP_09420777.1 ZP_09422525.1 ZP_09424253.1	503 476 428	556 503 489	450 413 384	467 517 531
Bradyrhizobium sp. S23321	YP_005448514.1	506	570	451	476
Bradyrhizobium sp. STM 3809	ZP_09426716.1 ZP_09430736.1	503 249	556	450	466 234
Bradyrhizobium sp. STM 3843	ZP_09439100.1 ZP_09434584.1	508 428	555 484	451 387	463 531
Bradyrhizobium sp. WSM1253	ZP_10082868.1	506	567	451	476
Bradyrhizobium sp. WSM471	ZP_09650765.1	506	567	451	476
Bradyrhizobium sp. YR681	ZP_10581440.1	506	570	451	476
Dinoroseobacter shibae DFL 12	YP_001532458.1	483	551	404	459
Erythrobacter litoralis HTCC2594	YP_459091.1	500	542	432	481
Erythrobacter sp. NAP1	ZP_01040540.1	452	523	403	448
Erythrobacter sp. SD-1	ZP_01864646.1	479	533	423	462
Hoeflea phototrophica DFL-43	ZP_02165873.1	511	560	448	474
Labrenzia aggregata IAM 12614	ZP_01545730.1 ZP_01550967.1	520 455	571 515	453 389	478 522
Labrenzia alexandrii DFL-11	ZP_05113600.1 ZP_05114288.1	519 456	562 519	448 399	470 514
Phaeobacter gallaeciensis 2.10	YP_006561816.1	442	495	400	442
Phaeobacter gallaeciensis DSM 17395	YP_006572046.1	442	495	400	442
Phaeospirillum molischianum DSM 120	ZP_09875084.1	502	550	420	489
Pseudovibrio sp. FO-BEG1	YP_005083198.1	524	569	454	488
Pseudovibrio sp. JE062 .	ZP_05086380.1	525	570	455	486
Rhodobacter capsulatus SB 1003	YP_003579323.1	442	487	399	443
Rhodobacter sp. AKP1	ZP_19197485.1	454	506	395	455

Bacterial Species	FtsH NCBI Accession	Similarity scores			
		360FtsH	703FtsH	417FtsH	745FtsH
Rhodobacter sp. SW2	ZP_05844627.1	468	507	397	466
Rhodobacter sphaeroides 2.4.1	YP_353740.1	454	506	395	455
Rhodobacter sphaeroides ATCC 17025	YP_001166777.1	454	503	392	454
Rhodobacter sphaeroides ATCC 17029	YP_001044192.1	454	506	395	455
Rhodobacter sphaeroides KD131	YP_002526382.1	448	507	394	455
Rhodobacter sphaeroides WS8N	ZP_08413447.1	454	506	395	455
Rhodobacteraceae bacterium HTCC2083	ZP_05074946.1	431	478	390	437
Rhodobacteraceae bacterium HTCC2150	ZP_01741361.1	452	501	397	448
Rhodobacteraceae bacterium KLH11	ZP_05121629.1	444	488	400	433
Rhodomicrobium vannielii ATCC 17100	YP_004013582.1 YP_004010946.1	533 436	581 483	446 403	482 483
Rhodopseudomonas palustris BisA53	YP_783598.1	506	571	458	480
Rhodopseudomonas palustris BisB18	YP_534584.1	508	568	452	482
Rhodopseudomonas palustris BisB5	YP_571261.1	507	568	455	476
Rhodopseudomonas palustris CGA009	NP_946477.1	510	574	459	483
Rhodopseudomonas palustris DX-1	YP_004107652.1 YP_004110651.1	510 472	574 536	456 408	483 545
Rhodopseudomonas palustris HaA2	YP_485437.1	509	571	454	479
Rhodopseudomonas palustris TIE-1	YP_001990332.1	510	574	459	483
Rhodospirillum centenum SW	YP_002298006.1	512	554	434	484
Rhodospirillum photometricum DSM 122	YP_005418704.1	516	572	439	495
Rhodospirillum rubrum ATCC 11170	YP_426186.1	509	563	433	501
Rhodospirillum rubrum F11	YP_006047398.1	509	563	433	501
Rhodovulum sp. PH10	ZP_10897193.1	499	571	460	476
Roseibium sp. TrichSKD4	ZP_07659890.1	513	566	452	474
Roseobacter denitrificans OCh 114	YP_682387.1	444	486	390	429
Roseobacter litoralis Och 149	YP_004690209.1	446	491	392	431
Roseobacter sp. AzwK-3b	ZP_01904150.1	457	511	398	450
Roseobacter sp. CCS2	ZP_01749336.1	451	494	403	429
Roseobacter sp. GAI101	ZP_05100730.1	431	481	389	430
Roseobacter sp. MED193	ZP_01057712.1	444	504	399	441

Bacterial Species	FtsH NCBI Accession	Similarity scores			
		360FtsH	703FtsH	417FtsH	745FtsH
Roseobacter sp. SK209-2-6	ZP_01752966.1	439	492	399	435
Roseovarius nubinhibens ISM	ZP_00958817.1	444	489	395	447
Roseovarius sp. 217	ZP_01037799.1	450	497	401	448
Roseovarius sp. TM1035	ZP_01879827.1	460	507	402	461
Ruegeria pomeroyi DSS-3	YP_168308.1	452	509	400	456
Ruegeria sp. R11	ZP_05088769.1	440	411	395	433
Ruegeria sp. TM1040	YP_614355.1	442	501	402	435
Ruegeria sp. TW15	ZP_08859903.1	439	494	403	434
Methylocystis sp. ATCC 49242	ZP_08074600.1 ZP_08074050.1 ZP_08074887.1 ZP_08075161.1	506 502 420 404	545 572 475 417	440 448 383 350	439 470 488 350
Novosphingobium sp. PP1Y	YP_004534092.1 YP_004534995.1 YP_004534997.1	500 454 429	530 509 481	439 388 374	481 436 493
***Betaproteobacteria* - Purple Non Sulfur Bacteria** [taxid: 28216]					
Rhodoferax ferrireducens T118	YP_523267.1 YP_523666.1	512 447	562 484	432 386	473 516
Rubrivivax benzoatilyticus JA2	ZP_08402255.1	499	544	421	461
Rubrivivax gelatinosus IL144	YP_005438161.1	498	543	421	461
Nitrosomonas europaea ATCC 19718	NP_840613.1 NP_840980.1	492 447	546 536	423 405	477 526
Ralstonia solanacearum CFBP2957	YP_003745416.1 YP_003748232.1	484 481	531 526	427 435	456 523
Gammaproteobacteria - Purple Sulfur Bacteria [taxid: 1236]					
Allochromatium vinosum DSM 180	YP_003442480.1 YP_003444220.1	509 501	573 570	426 409	493 470
Ectothiorhodospira sp. PHS-1	ZP_09695277.1	524	603	441	480
Halorhodospira halophila SL1	YP_001003336.1	503	559	438	475
Marichromatium purpuratum 984	ZP_08775190.1 ZP_08774973.1	514 507	572 570	436 417	500 469
Thiocapsa marina 5811	ZP_08770700.1 ZP_08772216.1	522 511	586 576	429 429	487 496
Thiocystis violascens DSM 198	YP_006414513.1 YP_006413098.1	504 489	577 560	433 413	492 456
Thiorhodococcus drewsii AZ1	ZP_08821484.1 ZP_08823620.1 ZP_08823254.1	518 445 500	575 513 566	429 431 429	481 468 500

Bacterial Species	FtsH NCBI Accession	Similarity scores			
		360FtsH	703FtsH	417FtsH	745FtsH
Thiorhodospira sibirica ATCC 700588	ZP_08922608.1	518	507	448	489
Thiorhodovibrio sp. 970	ZP_09869712.1	522	599	431	496
Escherichia coli DH1	YP_006090506.1 YP_006130468.1	511 511	592 592	438 438	486 486
Escherichia coli W	YP_006174806.1 ZP_07592743.1 YP_006126053.1	511 511 511	590 592 592	438 438 438	486 486 486
Actinobacteria [taxid: 201174]					
Streptomyces hygroscopicus subsp. jinggangensis 5008	YP_006246673.1 YP_006247848.1 YP_006241668.1	512 415 417	561 447 448	459 359 377	465 407 408
Patulibacter sp. I11	ZP_09468379.1 ZP_09467299.1 ZP_09467990.1	523 400 364	577 423 411	449 358 325	482 436 367
Deinococcus-Thermus [taxid: 1297]					
Meiothermus ruber DSM 1279	YP_003508253.1 YP_003506010.1	478 440	528 476	427 424	455 442
Deinococcus gobiensis I-0	YP_006262031.1 YP_006261779.1 YP_006271795.1	473 446 437	503 488 468	436 416 424	457 459 426
Oceanithermus profundus DSM 14977	YP_004058557.1 YP_004058516.1	453 420	505 450	410 388	455 420

4.4. Distribution of FtsH amongst eukaryotes

Notwithstanding that the focal point of this research project was the cyanobacterial FtsHases, looking how these proteases are distributed in the fascinating world of eukaryotes, never ceased to be less revealing or important. The curiosity driven research not being exclusive, adding details to the current picture of our understanding of how these proteases are distributed, evolve and function was an equally important driving force in this study.

With major structural and organizational differences at the cellular and organismal level between the two domains, it is important to understand how these, inherited from the bacterial world proteases have evolved and function in eukaryotes, at least in connection with the photosynthetic apparatus. Taking into consideration the general knowledge on

FtsHases as well as that on the operational model of their complex, and associating it with other information such as for instance the presence or absence of certain orthologs in particular organisms was proved worthwhile. Examination of such data provided important clues on the functional role(s) of particular FtsHases, and even whether the evolution of FtsH multigene-families parallels that of photosynthesis.

Looking for the distribution patterns of FtsH proteins among eukaryotes, the search, for the same reason as in bacteria, was entirely restricted to those major taxonomic groups that are known to contain photosynthetic species, albeit non-photosynthetic species within the group were often considered. Thus, the 'sequence to sequence' BLAST search was performed in the following nine major taxa: *Alveolata; Cryptophyta; Euglenozoa; Haptophyceae; Rhizaria; Rhodophyta; Stramenopiles-Heterokonts* and *Viridiplantae.*

Alveolata

In the phylum of *Alveolates,* despite the large number of hits, nearly 500 in just twenty organisms, the majority of the hits belonged to AAA family. With FtsHases not exceeding four per organism, the observed discrepancy between the true and false hits is attributable to the relatively small number of species that have been sequenced. The same phenomenon was observed in most of the examined phyla. However the *Alveolates* FtsH proteins, unlike any other examined in this study, were typified by longer primary structures, exceeding often 800 amino acid residues, Table VI.6.

Members of this monophyletic group are essentially unicellular organisms that have evolved various modes of nutrition, ranging from photoautotrophy to parasitism and predation, whereas in some lineages, e.g. *Chromera*, *Colponema*, Ellobiopsids, even that distinction between a predator and a parasite does not always fall into a clear-cut line. Most of the known *alveolates* belong into one of the four main subgroups: *Chromomerida, Dinflagelates, Ciliates* and *Apicomplexa.* While the study of FtsH presence in the former two classes is interesting because they both contain photosynthetic organisms, the study of the latter, famous for its notorious parasites such as *Plasmodium* causing malaria, is even more compelling because these species still possess plastids, or more accurately, remnant plastids, referred to as 'apicoplasts'. Knowledge of what type of FtsHases (if any) is present in these once phototrophs, that despite switching from photosynthesis to parasitism still contain 'plastids', may substantiate the implication of certain FtsHases in the maintenance of photosynthetic apparatus.

In contrast to *fts*H multigene families in non-photosynthetic species, the only two phototrophs, viz., *Durinskia baltica* and *Kryptoperidinium foliaceum* appeared with just a single FtsH, Table VI.6. However this picture is far from conclusive, not only because the whole genome sequences of the above two *alveolates* are still incomplete but mainly because a small sample of just twenty organisms, and that, mainly from the subgroup of *Apicomplexa,*is difficult to provide a definitive picture for such diverse phylum as *Alveolata.* Nevertheless, interpretation of the data in the view of particular characteristics of species was quite revealing.

The single FtsHases in the two photosynthetic dinoflagelates are encoded in their tertiary, diatom-derived chloroplasts (Imanian *et al*., 2010). And though lack of whole genome sequence prevents the acquisition of a more comprehensive picture, their similarity to FtsH2 is very informative, Table VI.6. Regardless whether other FtsH paralogs are encoded by any of their DNA sources (nucleomorphs, nucleous etc) the presence of FtsH2 in the chloroplast of the endosymbiont that is still separated by a single membrane from the host, suggests the importance of FtsH2 for the photosynthetic apparatus and is highly consistent with the accumulated so far knowledge.

The presence of FtsHases in multigene families in protists *Apicomplexa,* Table VI.6, while is indicative of their importance, the strong similarity to just FtsH5 via deductive reasoning also suggests the significance of FtsH2 to the photosynthetic apparatus inasmuch as the loss of the latter parallels the loss of photosynthetic ability overall.

Cryptophyta

Although *Cryptophyta* are ecologically and evolutionary important, lack of any significant research model or disease-causing organisms in this phylum, together with the multiplicity of their DNA sources resulted in small number of species that have been sequenced. The fact was reflected characteristically in the results with mere thirty hits in just four organisms Table VI.6. The data however were not as short of important information.

Inhabiting marine and freshwater environments, *Cryptophyta,* are unicellular, typically photosynthetic organisms, with a number of species still predating while being able to photosynthesize. With four DNA sources, i.e. nucleus, nucleomorph, mitochondrial and plastid, none of the four species in Table VI.6., has its entire genome sequenced. The results therefore discussed here are not the conclusive.

The only true FtsHases were identified in the photosynthetic species of *Rhodomonas salina* and *Guillardia theta.* Both proteases were chloroplast encoded and both exhibited characteristically stronger similarity towards FtsH2.

The nucleomorph and mitochondrial DNA of the third phototroph *Hemiselmi andersenii* contained no real FtsH. The non-photosynthetic but plastid containing *Cryptomonas paramecium,* was also found to have no real FtsH. Neither its three nucleomorph chromosomes, nor its plastid DNA appeared to contain any of *fts*H ORFs.

Based on certain premises, absence of any FtsH encoding genes, especially from the plastid DNA in *C. paramecium,* signifies the role of FtsH2 in TM housekeeping. Lateral *fts*H gene transfer from endosymbiont to host nucleus was not a universal phenomenon. FtsH encoding genes are found in the chloroplast genome of red & brown algae (Reith 1995; Kowallik 1995). All examined FtsHases in red algae were distinctly related to FtsH2, Table VI.6. Furthermore, the secondary chloroplast in *cryptohytes* is descended from a red algal cell. In conclusion, presence of *fts*H2 gene in chloroplast DNA of *Rhodomonas salina* and *Guillardia theta* on one hand side, and the absence of it from the plastid genome of *C. paramecium* coinciding with the loss of photosynthetic ability on the other, suggests the strong association of FtsH2 with the photosynthetic apparatus and probably their parallel evolution.

Euglenozoa

The phylum of *Euglenozoa,* a monophyletic group of singe-celled flagellate protozoans, is largely known for its parasitic members from the genera *Leishmania* and *Trypanosoma* causing leishmaniases and 'sleeping sickness' respectively. Photosynthetic species are restricted to the subgroup of *Euglenophytes* (taxid: 3035), that acquired the ability to photosynthesize through secondary endosymbiosis with a green algal cell (Leander *et al.,* 2001; Leander 2004). Search for FtsH homologs in this phylum was characterized by exceedingly large number of hits in relation to the number of organisms, 300 hits in just seven organisms, all parasitic from the genera of *Leishmania* and *Trypanosoma*. Although none of the hits was designated as FtsH, careful examination of the data showed that the top four hits in *Leishmania* and six in *Trypanosoma* species were in fact true FtsHases. (On occasions, especially when high throughput comprises the priority for the genomic data, scantily annotated entries may occur.) All high similarity hits were featured with regions characterizing FtsH proteins, i.e., name 'FtsH_fam' in TIGR database (The Institute of Genomic Research) and accession TIGR01241; and 'Peptidase_M41' in MEROPS database, or pfam01434 in Protein family (Pfam) Database. The 'Peptidase_M41' family has the *E.coli*

M41.001FtsH as the family type peptidase (MEROPS accession MER001620). The FtsH-nature of the top hits was cross-checked by BLAST. Taking as query some of the top-hits in *Leishmania* and *Trypanosoma,* homology searches against several bacterial and eukaryootic phyla were performed. The results, exclusively FtsH proteins, confirm that both genera contain *fts*H multigene families.

Glaucophyta & *Haptophyta*

Lack of complete genome sequences for any organism from the phyla of *Glaucophyta* and *Haptophyta* resulted in just a few hits of extremely low similarity Table VI.6. Nevertheless, future homology search in species such as *Cyanophora paradoxa,* a widely used model organism to study the development of chloroplasts, is undoubtedly worthwhile. With the cells retaining the blue-green appearance, having phycobilisomes as light harvesting antennas and most importantly with plastids that preserve a peptidoglycan layer between the inner and outer membranes of their envelop, these chloroplasts (cyanelles) are closer to the primary cyanobacterial endosymbiont as no other. Thus studying the presence, similarity distribution and the source of FtsH proteases (nucleus or chloroplast DNA) in this species may lead to some interesting findings and contribute to the current, overall picture of our understanding of FtsHases.

Rhizaria

The search for FtsH homologs among versatile and rich in species *Rhizaria* resulted in just five hits in two organisms, *Paulinella chromatophora* and *Bigelowiella natans* CCMP2755*,* Table VI.6. Although both species are photosynthetic, the origin of chloroplasts in them is very different. Result of primary endosymbiosis with a cyanobacterial cell in the former (for more details see above), and of secondary, derived from a green-algal cell in the latter. The single hit in *Bigelowiella natans* CCMP2755 was but a AAA-type protease. With nuclear DNA sequencing completed and the whole genome still in progress, BLAST on the database of the sequencing centre (JGI) revealed the presence of five FtsH homologs, Table VI.6. All appeared to be closely related to FtsH5. Nonetheless, as the sequences of chloroplast and nucleomorph DNA still not available no others conclusions could be made.

Rhodophyta

Red Algae (*Rhodophyta*) is one of the largest phyla of eukaryotic algae characterized by phycobiliproteins as accessory pigments and unstacked thylakoids in chloroplasts that have derived from primary endosymbiosis with a cyanobacterial cell. For those reasons as well as that the horizontal transfer of *fts*H genes from the symbiont to the host

nucleus has not been complete (Reith 1995), investigation of how these proteases are distributed in these organisms is of particular interest. The results show the presence of a single FtsH in each of the five identified species. It is noteworthy that all of them were invariably chloroplast encoded and belonged to FtsH2 type proteases, Table VI.6. Nonetheless, as the whole genome sequencing projects, for all five organisms, are still in progress, no other conclusions than the importance of FtsH2 for the maintenance of photosynthetic apparatus can be made.

Stramenopiles (Heterokonts)

Stramenopiles or *Heterokonts* are a major lineage of eukaryotic organisms. Metabolically and structurally versatile, including parasites, saprophytes, autotrophs and heterotrophs, with single-celled diatoms to multicellular giant kelps, and with the number of known species exceeding 100,000, this phylum is some of the largest in the domain of eukaryotes. The majority of *Heterokonts* are algae with secondary chloroplasts derived from red-algal cell (Cavalier-Smith 1999; Harper & Keeling 2003). The rest of *Stramenopiles* are colorless, lacking chloroplasts organisms. The most notable member of this group is *Phytophtora infestans* of *Oomycetes,* the cause of late blight in potatoes, that in the mid of 19th century was one of the major reasons of famine in Europe and Ireland, and caused, within a decade, over one million of people to starve to death.

The homology search in this phylum resulted in 175 hits in 15 organisms, almost all of which algae. The single non-photosynthetic organism, the notorious *P. infestans* contained more than thirty hits, albeit only three of them were true FtsHases, all closely related to FtsH5, Table VI.6. All brown and yellow-green algae appeared with a single FtsH, all of which were chloroplast encoded and closely related to FtsH2, Table VI.6. With none of these species having their entire genome sequenced, no other conclusion than the significance of FtsH2 proteases for the photosynthetic apparatus can be made at the present. The same results i.e. single, chloroplast encoded FtsH, closely related to FtsH2 were observed in all diatoms and the small, unicellular algae *Heterosigma akashiwo & Nannochloropsis gaditana* from the families of *Raphidiophyceae* and *Eusigmaceae* respectively. The chloroplast genome of diatoms have reduced number of genes compared to its red algal predecessor because of lateral gene transfer after the secondary endosymbiosis (Oudot-Le Secq *et al.,* 2007). Presence of *fts*H2 ORFs in chloroplast DNA of these widely distributed unicellular algae is yet another indication of FtsH2 important role in TM housekeeping.

Viridiplantae

The kingdom of *Viridiplantae* (Green Plants), with more than 350, 000 described species, that range in size, shape and structural organization, from microscopic unicellular algae to multicellular plants with sexual reproduction, is beyond doubt one of the largest and most diverse taxonomic clades. In recent years, the transition of organismal taxonomy from morphological to molecular differentiation has often caused (and maybe still causing) a pandemonium in our understanding of these groups. In the case of Green plants, the usage of the word plant is far from any conventional sense of the word, i.e. multicellular phototroph, and it is, a rather collective term, that alike 'plankton', reflects the ecological niche of the organisms it refers to and not the taxonomic classification.

Multiplication of *fts*H genes, and more so the evolution of FtsH2-type proteases are believed to correlate with the evolution of photosynthesis. Both events, multiplication and evolution of FtsH2-type are characteristically expressed in cyanobacteria, whereas in *Arabidopsis thaliana,* a well known green-plant species, the manifestation of the trend is even more emphatic. The homology searches in *Viridiplantae,* showed that the presence of a pair of highly related proteins for FtsH2 and FtsH5, is not restricted to *A. thaliana* only, but instead comprises a widely spread trait among Green plants. The 'sequence to sequence' BLAST against this kingdom generated more than 1000 hits in almost 30 species, Table VI.6. While multiple FtsH copies appeared to be a universal phenomenon in green plants, their presence in the group of Flowering plants, was emphatically higher, Table VI.6.

Table VI.7. Distribution of FtsH proteases among eukaryotes and similarity scores with the Synechococcus sp. 7942 FtsH proteins. The typographical representation with a strikethrough on a protein's accession number denotes that the particular protein does not correspond to a true FtsH. Asterisk in brackets at the end of organism's name refers to the sequencing status of the whole genome (nucleus, nucleomorph, mitochondrial, plastid) indicating that at least one DNA source has not been completely sequenced. Underlined organismal names designate non-photosynthetic species. The numbers next to the accession of each protein denote the length of the protein (amino acids). The numbers in square brackets correspond to the taxonomy ID for each taxa at NCBI Databases.

Eukaryotic organisms	FtsH NCBI Accession	Similarity scores			
		360FtsH	703FtsH	417FtsH	745FtsH
Alveolata [taxid: 33630]					
Durinskia baltica (*)	YP_003735018.1 / 625a.a	697	544	514	464
Kryptoperidinium foliaceum (*)	YP_003734582.1 / 627a.a	694	545	517	453
Babesia bovis T2Bo [ap]	XP_001611654.1 / 797a.a XP_001611107.1 / 658a.a XP_001609615.1 / 706a.a	402 366 364	420 393 376	372 350 316	387 390 361
Plasmodium falciparum 3D7 [ap]	XP_001348790.1 / 706a.a XP_001350791.1 / 880a.a XP_001347874.1 /1052a.a	395 366 375	413 414 399	337 342 351	365 389 375
*Cryptosporidium muris RN66 [pa]**	XP_002142291.1 / 775a.a	385	412	359	392
*Plasmodium chabaudi chabaudi [pa]**	XP_743809.1 / 850a.a	367	414	339	379
Theileria annulata str. Ankara [pa]	XP_952288.1 / 818a.a XP_953153.1 / 691a.a XP_953649.1 / 805a.a	394 374 338	407 370 357	370 309 329	376 344 341
Tetrahymena thermophila [ciliates]	XP_001007289.1 / 888a.a XP_001024476.1 / 884a.a XP_001470868.1 / 708a.a XP_001030952.2 / 741a.a	395 373 352 342	405 386 339 347	366 357 318 306	414 379 333 320
Plasmodium vivax Sal-1 [pa]	XP_001615738.1 / 702a.a XP_001615314.1 / 950a.a XP_001613933.1 / 896a.a	389 365 361	404 386 410	334 348 338	362 377 379
Plasmodium yield yield 17XNL [pa]	XP_724694.1 / 703a.a XP_725471.1 / 867a.a XP_726364.1 / 982a.a	387 366 368	409 411 399	333 335 346	362 379 377
Theileria parva str. Muguga [pa]	XP_764129.1 / 680a.a XP_766643.1 / 806a.a	382 338	380 360	317 327	351 340
Plasmodium berghei ANKA [pa]	XP_677920.1 / 769a.a XP_679159.1 / 849a.a	363 360	392 408	340 336	371 376
Cryptophyta [taxid: 3027]					
Rhodomonas salina (*)	YP_001293616.1 /628a.a.	944	650	603	461

Eukaryotic organisms	FtsH NCBI Accession	Similarity scores			
		360FtsH	703FtsH	417FtsH	745FtsH
Guillardia theta (*)	NP_050804.1 / 631a.a	934	646	609	465
Hemiselmis andersenii (*)	~~*XP_001712323.1*~~	188	198	182	176
Cryptomonas paramecium (*)	~~*XP_003239649.1*~~	195	198	189	185
Euglenozoa [taxid: 33682]					
Leishmania infantum JPCM5	XP_001469447.1 / 571a.a XP_001468475.1 / 623a.a XP_001465129.1 / 598a.a XP_001464878.1 / 598a.a	378 315 269 280	363 322 285 284	326 300 246 246	343 293 297 306
Trypanosoma cruzi str. CL Berner	XP_815566.1 / 657a.a XP_819897.1 / 657a.a XP_821024.1 / 712a.a XP_811493.1 / 712a.a XP_812938.1 / 683a.a XP_814657.1 / 682a.a	375 375 311 310 271 271	363 363 322 322 303 303	333 333 293 293 265 265	343 343 300 300 219 219
Glaucocystophyta [taxid: 38254]					
Cyanophora paradoxa (*)	~~*NP_043218.1*~~	28	31	21	20
Haptophyta [taxid: 2830]					
Emiliania huxleyi (*)	~~*YP_277323.1*~~	94	98	65	58
Phaeocystis antarctica (*)	~~*YP_005088638.1*~~	90	97	61	60
Rhizaria [taxid: 543769]					
Paulinella chromatophora	YP_002049185.1 / 615a.a YP_002048840.1 / 629a.a YP_002048793.1 / 620a.a	561 837 486	813 584 535	511 559 436	508 433 812
Bigelowiella natans CCMP 2755 (*)	~~*XP_001712900.1*~~ */ 527a.a* jig|Bigness|**52213**|estExt_Genewise1Plus.C_6017T jig|Bigness|52213|estExt_Genewise1Plus.C_6017T jig|Bigness|91991|estExt_fgenesh1_pg.C_1500003 jig|Bigness|86130|estExt_fgenesh1_pg.C_80149 jig|Bigness|88936|estExt_fgenesh1_pg.C_400146 jig|Bigness|141885|aug1.65_g16593	129 529 522 424 380 291 216	135 610 623 444 390 294 215	131 532 531 413 355 277 213	115 459 462 434 378 298 212
Rhodophyta [taxid: 2763]					
Porphyra purpurea	NP_053937.1 / 628a.a	941	640	612	477
Gracilaria tenuistipitata var. liui	YP_063571.1 / 626a.a	934	640	591	461

Eukaryotic organisms	FtsH NCBI Accession	Similarity scores			
		360Fts H	703Fts H	417Fts H	745Fts H
Pyropia yezoensi	YP_537009.1 / 628a.a	918	625	614	462
Cyanidioschyzon merolae str. 10D	NP_849040.1 /603a.a	751	583	564	464
Cyanidium caladium	**NP_045094.1** / 614a.a	749		568	456
Stramenopiles / Heterokonts [taxid: 33634]					
Odontella saneness [diatom]	NP_043642.1 / 644a.a	737	586	546	451
Thalassiosira pseudonana [diatom]	YP_874535.1 / 642a.a	725	566	536	452
Thalassiosira oceanica CCMP1005 (*) [diatom]	YP_004072526.1 / 642a.a	713	558	516	447
Phaeodactylum tricornutum (*) [diatom]	YP_874427.1 / 624a.a	709	541	521	446
Fistulifera sp. JPCC DA0580 (*) [diatom]	YP_004376591.1 / 626a.a	697	545	517	436
Synedra acus (*) [diatom]	YP_005089705.1 / 623a.a	697	561	518	442
Vaucheria litorea (*) [Yellow-Green algae]	YP_002327499.1 / 644a.a	736	544	526	422
Fucus vesiculosus (*) [Phaeophyceae]	YP_005090125.1 / 628a.a	627	512	467	406
Ectocarpus siliculosus (*) [Phaeophyceae]	YP_003289175.1 / 661a.a	612	509	459	395
Saccharina japonica [Phaeophyceae]	YP_006639120.1 / 628a.a	598	598	450	394
Heterosigma akashiwo	YP_001936449.1 / 663a.a	637	500	463	404
Nannochloropsis gaditana (*)	YP_007317003.1 / 697a.a	623	502	416	416
Phytophthora infestans T30-4 [Oomycetes]	XP_002902673.1 / 874a.a XP_002909608.1 / 658a.a XP_002898591.1 / 435a.a	414 401 282	454 433 305	391 359 253	405 363 277
Viridiplantae [taxid: 33090]					
Vitis vinifera (Grape Vine)	XP_002285925.1 / 706a.a XP_002282107.1 / 694a.a XP_002283393.2 /11146a XP_002283273.1 / 820a.a XP_002279005.2 / 709a.a XP_002268307.2 / 804a.a XP_002279721.1 / 818a.a XP_002266075.1 / 907a.a XP_002278786.1 / 888a.a XP_002279064.2 / 612a.a	583 746 704 404 392 398 363 360 313 284	756 564 656 421 414 404 380 323 319 286	493 518 530 363 343 329 345 320 291 259	483 467 457 413 379 357 426 281 261 263

Eukaryotic organisms	FtsH NCBI Accession	Similarity scores			
		360FtsH	703FtsH	417FtsH	745FtsH
*Brachypodium distachyon (*purple false brome*, monocot)*	XP_003563253.1 / 681α.α	576	749	490	473
	XP_003563254.1 / 676α.α	556	723	471	460
	XP_003563390.1 / 673α.α	733	562	513	456
	XP_003564049.1 / 669α.α	679	541	503	457
	XP_003567782.1 / 712α.α	396	421	356	383
	XP_003569240.1 / 704α.α	402	418	355	383
	XP_003568313.1 / 814α.α	401	416	364	410
	XP_003569373.1 / 767α.α	416	407	339	363
	XP_003559173.1 / 581α.α	377	401	346	345
	XP_003569989.1 / 811α.α	362	369	341	409
Medicago truncatula (Barrel Medic)	XP_003621234.1 / 671α.α	694	564	521	465
	XP_003603155.1 / 707α.α	401	423	349	379
	XP_003606687.1 / 807α.α	397	417	355	404
	XP_003624038.1 / 643α.α	391	412	351	401
	XP_003619575.1 / 765α.α	403	415	358	402
	XP_003616028.1 / 863α.α	380	401	332	365
	XP_003603156.1 /1307α.α	387	404	341	369
	XP_003615584.1 / 793α.α	373	381	343	419
	XP_003616029.1 / 689α.α	336	369	309	319
	XP_003615585.1 / 800α.α	358	368	327	404
	XP_003603157.1 / 668α.α	335	355	294	319
	XP_003597694.1 / 881α.α	298	326	288	261
	XP_003590119.1 / 510α.α	281	300	256	304
	XP_003615989.1 / 960α.α	273	290	231	249
	XP_003602591.1 / 883α.α	278	275	239	211
	XP_003628399.1 / 988α.α	239	276	222	197
Volvox carteri f. nagariensis (Gr.. algae)	XP_002948337.1 / 722α.α	583	743	497	478
	XP_002949662.1 /692α.α	747	582	534	453
	XP_002956225.1 / 640α.α	396	442	359	382
	XP_002952368.1 /1104α.α	381	389	329	362
	XP_002951779.1 /1105α.α	246	310	244	238
Chlamydomonas reinhardtii (Gr.. algae)	XP_001690889.1 / 727α.α	588	741	495	481
	XP_001697103.1 / 689α.α	758	581	529	453
	XP_001689949.1 / 752α.α	389	401	369	386
Micromonas pusilla CCMP1545 (Gr. algae)	XP_003057154.1 / 731α.α	575	733	504	468
	XP_003064115.1 / 613α.α	400	431	360	372
	XP_003059742.1 / 651α.α	401	424	365	399
	XP_003055147.1 / 941α.α	383	389	347	367
	XP_003055580.1 / 571α.α	344	372	316	384
Physcomitrella patens subsp. patens (Mosses)	XP_001753657.1 / 647α.α	570	743	478	472
	XP_001760664.1 / 634α.α	569	740	478	470
	XP_001772895.1 / 696α.α	764	589	529	489
	XP_001753506.1 / 688α.α	760	582	526	478
	XP_001774532.1 /630α.α	732	580	515	458
	XP_001769853.1 / 635α.α	754	573	528	453
	XP_001756660.1 / 677α.α	405	432	358	383
Selaginella moellendorffii (Club-mosses)	XP_002960384.1 / 628α.α	562	736	493	466
	XP_002967332.1 / 628α.α	562	736	493	466
	XP_002979030.1 / 595α.α	754	609	520	466
	XP_002984945.1 / 595α.α	752	606	521	467
	XP_002974515.1 / 597α.α	723	566	508	458
	XP_002990783.1 / 691α.α	734	563	512	461
	XP_002981661.1 / 737α.α	393	437	360	417

5. FtsH proteins in cyanobacteria

Search for FtsH homologs in our model organisms, revealed the presence of *fts*H multigene families in each of them, yet the multiplication of these proteases was associated with the evolution of oxygenic photosynthesis. Nevertheless as our model organisms are relatively closely related species, while cyanobacteria constitute a large and diverse group of organisms found in almost every conceivable habitat; to investigate whether the presence of four FtsHases is not a random event restricted to some akin species and also to understand whether the multiplication of FtsHases is associated with the evolution of photosynthesis, and why not to quench the thirst of our curiosity, homology searches against the phylum of cyanobacteria were performed.

All organisms, one hundred and twenty species from 38 genera appeared to contain several FtsHases, ranging from just two copies per organism in *Synechococcus sp.* JA-2-3B'a(2-13) and JA-3-3Ab, (Table VI.7. A, B) to the really impressive nine copies in *Acaryochloris marina* MBIC11017 and *Acaryochloris sp*. CCMEE 5410, (Table VI.7. A, B). The majority of species however, at least 90% of them appeared to contain four or more of these proteases. The trait of four FtsHases per organism, albeit the predominant, was not as universally conserved as in our model organisms. If the truth to be told, universality of four FtsHases per organism in the diverse world of cyanobacteria would rather be very surprising. The presence of four FtsH proteins in each cyanobacterium was not even conserved in the genera of our model organisms, i.e. *Synechocystis* and *Synechococcus*, were strains with three and five FtsH proteins respectively were identified, Table VI.7.A.B. Existence of different number of FtsHases in species from the same genera, e.g. *Anabaena*, *Arthrospira*, *Cyanothece*, *Leptolyngbya*, along with the aforementioned ones, discloses, if nothing else, the great plasticity of these ancient organisms. However, holding the accent of this investigation on the precise number of FtsHases rather than on their multiplicity would certainly be less constructive and maybe spurious. Instead, the conclusion to be stressed is that the trait of multiple copies is not confined to just a few species but characterises cyanobacteria regardless of their morphological, taxonomical, ecological and physiological attributes. Yet, the very existence of *fts*H multigene families, in unicellular (e.g. *Microcystis)* or colonial species (e.g. *Arthrospira),* in species that either divide by binary (e.g. *Synechococcus*) or multiple fission (e.g. *Xenococcus)*, or by budding (e.g. *Chaimosiphon),* or in one or many successive planes (e.g. *Gleocapsa*), that differentiate into heterocystous filaments (e.g. *Nostoc)* or not (e.g. *Lyngbya*), and so on, signifies that multiplicity of these proteases in cyanobacteria is more likely to be the result of a

feature that they all have in common rather than differentiate them into varying taxa. In other words, multiplication of FtsH proteases in cyanobacteria seems to be associated with the evolution of photosynthesis in these organisms.

As the multiplicity of FtsHases seems to typify the phylum of cyanobacteria and yet to be associated to certain degree with the evolution of oxygenic photosynthesis, the next step of the investigation was to understand whether the formerly described relationships between the FtsHases in *A. thaliana* and in our model organisms characterise the rest of cyanobacteria. To acquire such insights, the similarity scores of all FtsHases were analysed and orthologous proteins were identified, Table VI.7.C.

Examination of cases with two or three FtsHases, in spite of being limited to just a few organisms, was very informative as far as the *modus operandi* of the holoenzyme and the evolutional pattern of these proteases in cyanobacteria are conserved.

Thus the instance of two FtsHases in one organism, although of limited frequency of occurrence, just two *Synechococcus* strains (Table VI.7.), and ecologically confined to the octopus thermal spring in Yellowstone National Park (USA), is very interesting for very different reasons. Unlike other nitrogen fixing cyanobacteria, both strains JA-2-3Ba(2-13) and JA-3-3Ab, were the first reported species capable of performing the metabolic tasks of oxygenic photosynthesis and nitrogen fixation (Steunou *et al.,* 2006; Devaki *et al.,* 2007) within a single cell without forming specialised for the fixation heterocysts. In regard to the presence of just two FtsHases, the case is of particular interest to seek answers on the operational model of FtsH-complex. Considering the multimeric nature of the enzyme and the presence of just two FtSHases in these ecotypes, irrespective of whether they are the result of deletion or the first step in multiplication, this case is unique because it allows to evaluate how essential is the presence of two types of FtsHases, A & B, for the formation of a functional holoenzyme in oxygenic phototrophs.

Given that the phylum of Cyanobacteria is dominated by species with four or more FtsHases (in excess of 90%), organisms with three FtsH proteins are of particular interest to acquire important insights on evolution pattern of these proteases in the clade.

Examination of the results, Table VI.7.C, revealed that all cyanobacteria, regardless of the number of FtsHases they possess, two or nine, they always contain one single copy of FtsH5. The fact stresses the significance of this protease and correlates highly with

the experimental data showing the vital role of this protease for cell homeostasis and survival. The presence of a sole copy of FtsH5 emphasises even further its importance, because as the archetypal form it plays the role of a typing matrix, of a template that the rest of FtsHases are produced. Duplicates once produced are free to change and evolve new structures and functions, but the original template for each organism has to be conserved.

Nonetheless, there seems to be an exception to the rule of a single FtsH5 per organism. This is the case of *Acaryochloris sp.* CCMEE 5410, a cyanobacterium with the impressive nine FtsH copies, three of which appeared to be closely related to FtsH5, Table VI.7.B&C. Application in this instance of the proverb 'exception confirms the rule', a phrase that is already frequently misused, would rather state that exceptions should be accepted or / and neglected, yet more their presence strengthens the rule. Instead of following this path, the seeming exceptions were scrutinised and because the definition of FtsH5 appeared not to apply to these exceptions, the rule of a single FtsH5 per organism seems to be valid after all. When comparing the similarity score of one protein to a set of other homologs, higher score to a particular protein does not make these particular proteins automatically orthologous, especially when this score is well outside the score lines of established orthologies. In other words, comparison of similarity scores of one protein to more than two will inevitably show some scores to be higher than others, but that does not and should not automatically qualify the pair of proteins as orthologous. Thus, the ZP_09246381.1 protein, with 902 maximum score towards FtsH5 is very likely to be the orthologous FtsH, while the other two putative FtsH5 proteases, viz., ZP_09250913.1 & ZP_09246799.1, exhibiting substantially lower scores (536 and 308 respectively) appear to be rather homologous duplicates that may be in process of evolving new functions, especially when considering the overall number of FtsHases in this organism. The results of MSA and the produced phylogenetic tree, Figure VI.2, showing the ZP_09250913.1 protein (named as Ac54-5s) clustering within the group of 745-like FtsHases instead of FtsH5, suggests that the similarity score relating it to FtsH5 may have occurred by chance. Furthermore, the other protein seemingly closer to FtsH5, viz; ZP_09246799.1, with only 327 amino acids residues and having no clearly defined AAA domain, annotated therefore as partial FtsH, is very unlikely to be a true FtsH5 ortholog.

The results displayed in Table VI.7.C, disclosed yet another important feature of FtsH distribution among cyanobacteria. All examined species, regardless of their

morphological or ecological characteristics, or even of the total number of FtsHases they possess, they all contain Type-B FtsHases. Almost invariably this Type-B of proteases were represented by two closely related but distinct proteins, either both closely related to FtsH2 or one to FtsH2 and the other to 417-like proteins. At this point however, it must be elucidated that in all species where two of their FtsH proteins were exhibiting higher similarity towards FtsH2 e.g. *Microcystis aeruginosa PCC 9806 (*ZP_16390426.1 ZP_16390809.1); *Nostoc* sp. PCC7524 (YP_007074951.1 YP_007077799.1) only one them with the notably higher similarity score seemed to be truly FtsH2 . The second one, although ascribed to FtsH2 group, was exhibiting nearly the same scores towards both FtsH2 and 417FtsH, but slightly higher towards FtsH2. For these reasons, when analyzing the distribution pattern of similarity scores and constructing phylogenetic trees, the second FtsH2-like protein (exhibiting the lower score), to be distinguished from both FtsH2 and 417- like proteins was designated as FtsH2s (where 's' standing for second).

Although the described above canon of two Type-B FtsH proteins in each organism was plainly evident in all examined species and strains, there were nevertheless some cases that seemed to contradict this rule.

The first 'deviation' from the rule was identified in the two *Synechococcus* ecotypes isolated from Yellowstone thermal spring. Both strains contain one instead of two Type-B FtsHases. However, as the number of these proteases in these isolates is restricted to just two copies per organism, one of which is the archetypal form (FtsH5), the presence of just one Type-B protein then, simply confirms the existence of the rule dictating the necessity of Type-B FtsHases coexisting or complementing FtsH5.

Another exception seemed to be the species of *Gloeobacter violaceus* PCC 7421, that in spite of containing four FtsH homologs, only one them was of Type-B, Table VI.7. B & C. This organism contain one copy of FtsH5 and FtsH2, and two of 745-like proteins, a pattern of distribution observed in no other cyanobacterium with four FtsHases. In high contrast however to any known cyanobacterium, *Gloeobacter violaceus* contain no thylakoid membranes. Lack of second Type-B FtsHases, either FtsH2 or 417-like, from an organism without TM, might suggest that the evolution of the second Type-B protease in cyanobacteria is somehow associated with the development of TM. Despite being a mere speculation, this hypothesis however has certain experimental ground for further investigation. While the cyanobacterial FtsH2 is involved in TM quality control, but does not affect the normal development of these membranes, the 417-like FtsHases

are essential for the cell; possibly because of their role in TM ontogenesis. Investigation for instance how an insertional mutagenesis of 417-like *fts*H gene in cyanobacterium capable of growing heterotrophically (e.g. *Synechocystis*), will affect the development of thylakoid membranes will certainly provide important information on the functional role of 417-FtsHases.

The second 745-like FtsHases (accession NP_925595.1) in *Gloeobacter violaceus* PCC 7421, with 785 a.a residues, despite being considerably longer than the rest of cyanobacterial FtsHases, is a protein of low overall similarity, Table VI.7.B. It is plausible therefore that the slight inclination towards the group of auxiliary FtsHases has rather occurred by chance and do not represent any kind of orthology. The phylogenetic tree of FtsHases, Figure VI.2, showing clearly this protein clustering separately, correlates well with the suggestion of arbitrary similarity.

The third seeming aberration from the requirement of two Type-B FtsHases per organisms is the species of *Arthrospira platensis str. Paraca,* that contain six FtsH proteins, Table VI.7.A,B. This particular organism appear with three Type-B FtsHases (one FtsH2 and two 417-like FtsHases) instead of two. The second 417-like FtsHases, accession ZP_11273220.1, is again a protein of low overall similarity, and because of that it is difficult to certify whether it is a true 417-like FtsH or the score occurred by chance. Yet more, with only 249 a.a. residues, and lacking the WalkerB motif from its AAA domain (that alone in FtsHases is 200-250 a.a.) this protein was described as partial FtsH. Nonetheless, if this protein is a true Type-B FtsH, its presence might be associated with the high rates of oxygen evolution characterizing this organism (Ciferri 1983).

Another important characteristic of FtsH distribution in cyanobacteria relates to 745-like FtsHases. This particular group of proteases exists exclusively in species with four or more FtsHases and it is noteworthy that when the number of FtsHases in an organism increases beyond four copies, then any further multiplication of FtsHases is invariably of 745-like type. Protein's function is subject to evolution, so proteins that are homologous and have related sequences or / and structures, sometimes may have different functions. Gene duplication or swapping and recombination of modular units (domains) are thought to consist the major route for the evolution of new and more complex protein functions.

Complete absence of this group of proteases from species with two or three FtsHases on one hand side, and their functional redundancy in species with four on the other, e.g. *Synechocystis* 6803 (Mann *et al.,* 2000), along with the above-stated conclusions, disclose that the basic requirement for formation of a functional FtsH-complex is the presence of one FtsH5 and two Type-B FtsHases one of which at least must be FtsH2. Although the 745-like FtsHases do not seem to be directly involved in any important functional role, at least easily detectable upon deletion of the respective gene, their multiplication nevertheless is unlikely to occur in vain. This type FtsHases therefore is plausible to play auxiliary role(s) under specific conditions and requirements. For these reasons this particular category of proteins hereafter will be referred as the auxiliary group and denoted as FtsH-Ax.

Table VI.8. **Distribution of FtsH proteins in Cyanobacteria. A)** Cyanobacterial species and the number of FtsHases present in each organism. The numbers in square brackets designate the taxonomy ID for each organism at NCBI databases. **B)** Similarity scores between the query FtsHases and those in selected cyanobacteria. **C)**. Types of FtsHases identified in 120 cyanobacteria, and frequency of occurrence. Asterisks in this part refer to particular cases, reflected in detail at the end of the table.

A	**Cyanobacteria with 2 FtsHases**
Synechococcus sp. JA-2-3B'a(2-13) [321332]; JA-3-3Ab [321327]	
Cyanobacteria with 3 FtsHases	
Anabaena sp. 90 [46234]	
Cyanobacterium aponinum PCC 10605 [755178]	
Cyanobacterium stanieri PCC 7202 [292563]	
cyanobacterium UCYN-A [713887]	
Dactylococcopsis salina PCC 8305 [13035]	
Geitlerinema sp. PCC 7407 [1173025]	
Oscillatoria acuminata PCC 6304 [56110]	
Synechocystis sp. PCC 7509 [927677]	
Cyanobacteria with 4 FtsHases	
Anabaena cylindrica PCC 7122 [272123]	
Anabaena variabilis ATCC 29413 [240292]	
Arthrospira maxima CS-328 [513049]	
Arthrospira platensis C1 [459495]	
Arthrospira sp. PCC 8005 [376219]	
Calothrix sp. PCC 6303 [1170562]; PCC 7507 [99598]	
Chroococcidiopsis thermalis PCC 7203 [251229]	
Coleofasciculus chthonoplastes PCC 7420 [118168]	
Crinalium epipsammum PCC 9333 [1173022]	
Crocosphaera watsonii WH 0003 [423471]; WH 8501 [165597]	
Cyanobium gracile PCC 6307 [292564]	
Cyanobium sp. PCC 7001 [180281]	

Cyanothece sp. ATCC 51142 [43989]*;* ATCC 51472 [860575]*;* CCY0110 [391612]*;* PCC 7425 [395961]

Cylindrospermopsis raciborskii CS-505 [533240]

Cylindrospermum stagnale PCC 7417 [56107]

Gloeobacter violaceus PCC 7421 [251221]

Leptolyngbya sp. PCC 6406 [1173264]*;* PCC 7376 [111781]

Microcoleus sp. PCC 7113 [1173027]

Microcoleus vaginatus FGP-2 [756067]

Moorea producens 3L [489825]

Nostoc azollae' 0708 [551115]

Nostoc punctiforme PCC 73102 [63737]

Nostoc sp. PCC 7107 [317936]*; PCC 7120 [103690]*

Oscillatoria nigro-viridian PCC 7112 [179408]

Oscillatoria sp. PCC 6506 [272129]

Oscillatoriales cyanobacterium JSC-12 [864702]

Prochlorococcus marinus str. AS9601 [146891]

Prochlorococcus marinus MIT 9202 [93058]*;* MIT9211 [93059] *;* MIT9215 [93060]*;* MIT9301 [167546] *;* MIT9303 [59922] *;* MIT9312 [74546] *;* MIT9313 [74547]*;* MIT9515 [167542]*;* NATL1A [167555]*;* NATL2A [59920]*;* CCMP1375 [167539]*;* CCMP1986 [59919]

Pseudanabaena biceps PCC 7429 [927668]

Pseudanabaena sp. PCC 7367 [82654]

Raphidiopsis brookii D9 [533247]

Synechococcus elongatus PCC 6301 [269084]*;* PCC 7942 [1140]*;* BL107 [313625]*;* CB0101 [232348]*;* CB0205 [232363]*;* CC9311 [64471]*;* CC9605 [110662]*;* CC9902 [316279]*;* PCC6312 [195253]*;* PCC7002 [32049]*;* PCC7502 [1173263]*;* RCC307 [316278]*;* RS9916 [221359]*;* RS9917 [221360]*;* WH5701 [69042]*;* WH7803 [32051]*;* WH7805 [59931]*;* WH8016 [166318]*;* WH8102 [84588]*;* WH8109 [166314]

Synechocystis sp. PCC 6803 [1148]*; substr.* GT-I [1080228]*; substr.* PCC-N [1080229]*; substr.* PCC-P [1080230]

Thermosynechococcus elongatus BP-1 [197221]
Trichodesmium erythraeum IMS101 [203124]
Xenococcus sp. PCC 7305 [102125]
Cyanobacteria with 5 FtsHases
Chamaesiphon minutus PCC 6605 [1173020]
Cyanothece sp. PCC 7424 [65393]; PCC 7822 [497965]; PCC 8801 [41431]; PCC 8802 [395962]
Fischerella sp. JSC-11 [741277]
Gloeocapsa sp. PCC 73106 [102232]; PCC 7428 [1173026]
Halothece sp. PCC 7418 [65093]
Lyngbya sp. PCC 8106 [313612]
Microcystis aeruginosa NIES-843 [449447]; PCC 7941 [213618]; l PCC 9432 [1160280]; PCC 9443 [1160281]; PCC9701 [721123]; PCC 9717 [1160286]; PCC 9806 [1160282]; PCC 9807 [1160283]; PCC 9808 [1160284]; PCC 9809 [1160285]; TAIHU98 [1134457]
Microcystis sp. T1-4 [1160279]
Nodularia spumigena CCY9414 [313624]
Nostoc sp. PCC 7524 [28072]
Rivularia sp. PCC 7116 [373994]
Stanieria cyanosphaera PCC 7437 [111780]
Synechococcus sp. PCC 7335 [91464]
Cyanobacteria with 6 or more FtsHases
Arthrospira platensis str. Paraca [634502] (6 FtsHases)
Leptolyngbya sp. PCC 7375 [102129] (7 FtsHases)
Pleurocapsa sp. PCC 7327 [118163] (*7* FtsHases)
Acaryochloris marina MBIC11017 [329726] (9 FtsHases)
Acaryochloris sp. CCMEE 5410 [310037] (9 FtsHases)

B Cyanobacteria	FtsH NCBI Accession	Similarity scores (Max score)			
		FtsH2	FtsH5	417FtsH	745FtsH
Acaryochloris sp. CCMEE 5410	ZP_09250667.1 ZP_09249363.1 ZP_09246381.1 ZP_09250913.1 ZP_09247862.1 ZP_09252909.1 ZP_09246925.1 ZP_09248739.1 ZP_09246799.1	956 661 618 502 332 306 301 289 286	656 575 902 536 345 330 314 305 308	599 693 541 457 297 300 289 258 254	447 421 494 508 364 503 349 403 205
Arthrospira platensis str. Paraca	ZP_11277156.1 ZP_11275722.1 ZP_11274485.1 ZP_11276078.1 ZP_11273220.1 ZP_11274430.1	1058 656 474 281 211 208	651 982 402 317 189 221	646 566 501 259 220 180	516 558 312 473 137 430
Cyanobacterium aponinum PCC 10605	YP_007162069.1 YP_007161923.1 YP_007162923.1	1018 748 643	647 606 952	626 635 560	527 505 556
Gloeobacter violaceus PCC 7421	NP_925524.1 NP_924863.1 NP_926087.1 NP_925595.1	814 636 507 281	658 843 535 278	590 567 459 275	472 496 619 300
Microcystis aeruginosa PCC 9806	ZP_16390426.1 ZP_16390809.1 ZP_16391961.1 ZP_16390228.1 ZP_16389001.1	1041 723 642 497 476	661 617 965 544 507	624 628 556 445 431	506 488 547 931 709
Nostoc punctiforme PCC 73102	YP_001865009.1 YP_001865348.1 YP_001868170.1 YP_001865592.1	1029 790 643 495	676 635 956 551	640 660 560 450	473 465 500 807
Nostoc sp. PCC 7524	YP_007074951.1 YP_007077799.1 YP_007076726.1 YP_007076914.1 YP_007075185.1	1036 794 643 513 499	679 647 959 556 544	652 667 567 456 436	519 514 551 707 688
Oscillatoria acuminata PCC 6304	YP_007085438.1 YP_007084560.1 YP_007083980.1	1022 687 645	654 581 966	641 744 565	510 468 545
Rivularia sp. PCC 7116	YP_007056358.1 YP_007056207.1 YP_007053357.1 YP_007054408.1 YP_007058059.1	1040 694 646 514 489	675 588 953 556 534	638 702 568 453 450	513 483 543 724 664
Synechococcus ps. JA-2-3B'a(2-13)	YP_476269.1 YP_476882.1	740 636	610 821	756 605	504 535
Synechococcus sp. JA-3-3Ab	YP_473500.1 YP_476117.1	726 631	607 808	752 599	501 538
Synechococcus sp. PCC 7335	ZP_05038188.1 ZP_05035182.1 ZP_05034981.1 ZP_05036551.1 ZP_05039862.1	991 640 634 509 451	644 932 542 567 502	615 585 636 460 407	473 511 429 763 584

B Cyanobacteria	FtsH NCBI Accession	Similarity scores (Max score)			
		FtsH2	FtsH5	417FtsH	745FtsH
Synechocystis sp. PCC 7509	ZP_21041137.1 ZP_21041249.1 ZP_21039168.1	1022 758 625	659 610 952	610 643 558	493 494 546

C	Type-A	Type-B		Auxiliary	Number of species with the observed pattern
	FtsH5	FtsH2	417FtsH	745FtsH	
2 FtsHases per organism	1	-	1*	-	2
3 FtsHases per organism	1	2	-	-	5
	1	1	1	-	3
4 FtsHases per organism	1	1**	-	2	1
	1	2		1	28
	1	1	1	1	28
5 FtsHases per organism	1	2		2	21
	1	1	1	1	5
6 FtsHases per organism	1	1	2***	2	1
7 FtsHases per organism	1	1	1	4	1
	1	2		4	1
9 FtsHases per organism	1	1	1	6	1
	3****	1	1	4	1

*The case of two *Synechococcus* strains **(**JA-2-3B'a(2-13) JA-3-3Ab) found in octopus thermal spring in Yellowstone National Park, USA. The single type-B-FtsH identified in these species, unlike other species, seems to slightly closer to 417FtsH rather than to FtsH2. Nonetheless it is clearly a Type-B FtsH protein.

** The only species with a single Type-B FtsH is ***Gloeobacter violaceus,*** a unique cyanobacterium that contains no thylakoid membranes.

*** *Arthrospira platensis str. Paraca* with six FtsHases. Unlike the rest of cyanobacteria, this species seems to contain two 417-like proteins and one FtsH2. Closer examination however reveals that the second 417-like FtsH, (accession ZP_11273220.1) is a protein of very low similarity to all four query sequences.

**** This the case of *Acaryochloris sp. CCMEE 5410* which encodes nine FtsHases. This the only organism that in high contrast to the rest of the examined species appeared to contain three FtsH5 proteins instead of one, viz., 1) ZP_09246381.1; 2) ZP_09250913.1; 3) ZP_09246799.1. Unlike the first one that exhibits distinct high similarity to FtsH5, the other two show considerably lower levels of similarity, that no other FtsH from all 120 species have shown towards FtsH5.

5.1. Phylogenetic analysis of cyanobacterial FtsH proteins

Analysis of similarity scores showed that the FtsH proteins in cyanobacteria, regardless of their total number in an organism, seemed to evolve according to certain model. It was shown that there is invariably a single FtsH5 that is always accompanied by two Type-B FtsHases, one of which is FtsH2. These three proteins seemed to form an indispensable core complemented by a group of auxiliary FtsHases that its number in each organism depends on the total number of these proteases. To investigate this hypothesis further and understand deeper the phylogenetic relationships between FtsH proteins in cyanobacteria, inescapably a MSA and construction of phylogenetic tree were performed.

For the purposes of such analysis a large number of FtsHases (167) from various cyanobacteria was selected. To be as representative of the phylum as possible the chosen sample of 40 organisms, contained species and strains from all cyanobacterial lineages and with all described numbers of FtsHases. Inclusion in some cases of two or more species from the same genus was founded either upon existence of different number of FtsH proteins in these organisms, e.g. *Cyanothece sp*. ATCC 51142 with four and PCC 7424 with five FtsHases, or to represent species from different habitats, especially from diverse genera such as *Synechococcus* (*Honda et al.*, 1999) e.g. marine *Synechococcus sp.* WH5701 and fresh water PCC7942. The majority of omitted species are different isolates of one species, e.g., *Microcystis aeruginosa, Prochlorococcus marinus,* Table VI.7. A.

As an out-group protein, the sequence of FtsH (YP_006262031.1) in *Deinococcus gobiensis* I-0 (phylum Deinococcus-Thermus) was used.

All 168 FtsH protein sequences were aligned with Clustal Omega (the newest version of ClustalW) software using the server at EMBL-EBI with default parameters chosen. A Gonnet matrix was used to generate the protein sequence distance matrix that was used in the phylogenetic analysis employing the neighbour-joining (NJ) method (Saitou & Nei, 1987). Support for nodes was estimated by boot-strapping of 1000 data re-sampling. For visual representation of the trees the software SplitsTree4 (Huson & Bryant 2006) and FigTree were used.

Most of current programs for multiple alignments use the method of progressive alignment, which by making a preliminary assessment of how the sequences are related using pair-wise alignments, construct a guide tree and then based on that add

progressively sequences starting with the most closely related ones and finishing with the most distant one. Although advantageous of being relatively fast, suffer from the problem that errors made early in the process can never be rectified. Therefore, in order to obtain the best possible results, all 168 sequences before the alignment were grouped by decreasing similarity order known from BLAST searches. Yet, before the construction of any phylogenetic tree, the produced MSA was manually checked whether the amino acid residues within Walker A & B motifs and the catalytic site were correctly matched. To perform this examination the program Jalview was used. It is an excellent tool designed for visualisation of large MSA files. Jalview is an open source project developed at the University of Dundee's College of Life Sciences.

With 168 sequences analysed, all protein names and the species they belong to, were for readability reasons truncated. Naming the proteins with their accessions numbers was excluded as it would rather bring more confusion than clarity. For ease of reference, abbreviation of each protein and the name of the organism it belonged to was based on certain set of rules. Each abbreviation was composed of two parts separated by a dash. The first part reflects the organism's name and the alphanumerical designation of particular isolate (culture). The second, numerical part represents the type of FtsH that each proteins belongs to (FtsH2, FtsH5, 417, Ax). In particular, when the full species name was known, e.g. *Microcystis aeruginosa*, then the initials of the genus and species names were used, viz., 'Ma', followed by the first two letters or digits of the alphanumerical name of the collection. Thus for instance, in the above case of *Macrocystis aeruginosa,* the two selected cultures, NIES-843 & PCC 9806 were finally abbreviated to MaNI and Ma98 respectively. When the initials of the culture collection were the same, e.g.. PCC (Pasteur Culture Collection), then the first two digits were used instead. Thus, *Synechocystis sp*. PCC680 and *Synechocystis sp*. PCC7509 were ultimately reduced to Ss68 and Ss75 respectively. The second part of the truncated name was a numerical representation of the type of FtsH that each protein belonged to. Thus for instance in *Microcystis aeruginosa PCC9806 the five FtsHases* it contains were named as, Ma98-5 denoting FtsH5 in this organism; Ma98-2 denoting FtsH2; Ma98-2s denoting Type-B protein with slightly higher score towards FtsH2 than to 417FtsH; Ma98-Ax denoting an FtsH from the auxiliary group. In species with more than one Auxiliary FtsHases (e.g. *Acaryochloris marina* MBIC11017), discrimination of each of Auxiliary protein was based on a numerical suffix e.g. Ax1, Ax2 etc. that was chosen according to their similarity scores towards 745FtsHases. For more details however on the species used and the abbreviated names of their proteins see at Table VI.8.

Table VI.9. **Cyanobacterial species used for the construction of phylogenetic tree and the abbreviated names of their FtsH proteins.**

Organism	abbreviation	Abbreviated protein names
Acaryochloris marina MBIC11017	Acm	Acm-5; Acm-2; Acm-2s; Acm-417; Acm-Ax1 -5
Acaryochloris sp. CCMEE 5410	Ac54	Ac54-5; Ac54-5s; Ac54-2; Ac54-417; Ac54-Ax1-2
Anabaena cylindrica PCC 7122	Ancy	Ancy-5; Ancy-2; Ancy-2s; Ancy-Ax
Anabaena sp. 90	An90	An90-5; An90-2; An90-2s; Ancy-Ax
Arthrospira platensis str. Paraca	ArpP	ArpP-5; ArpP-2; ArpP-417; ArpP-Ax1-2
Arthrospira sp. PCC 8005	Ar80	Ar80-5; Ar80-2; Ar80-417; Ar80-Ax
Chamaesiphon minutus PCC 6605	Chm	Chmm-5; Chmm-2; Chmm2s; Chmm-Ax
Chroococcidiopsis thermalis PCC 7203	Chth	Chth-5; Chth-2; Chth-417; Chth-Ax
Cyanobacterium aponinum PCC 10605	Cybap	Cybap-5; Cybap-2; Cybap-2s
Cyanothece sp. ATCC 51142	CyAT	CyAT-5; CyAT-2; CyAT-2s; CyAT-Ax
Cyanothece sp. PCC 7424	CyPC	CyPC-5; CyPC-2; CyPC-2s; CyPC-Ax1; CyPC-Ax2
Dactylococcopsis salina PCC 8305	Dacs	Dac-5; Dac-2; Dac-417
Fischerella sp. JSC-11	Fis	Fis-5; Fis-2; Fis-417; Fis-Ax1; Fis-Ax2
Geitlerinema sp. PCC 7407	Geit	Geit-5; Geit-2; Geit-417
Gloeobacter violaceus PCC 7421	Glv	Glv-5; Glv-2; Glv-Ax1; Glv-Ax2
Leptolyngbya sp. PCC 6406	Lp64	Lp64-5; Lp64-2; Lp64-417; Lp64-Ax
Leptolyngbya sp. PCC 7375	Lp73	Lp73-5; Lp73-2; Lp73-417; Lp73-Ax1-4
Microcystis aeruginosa NIES-843	MaNI	MaNI-5; MaNI-2; MaNI-2s; MaNI-Ax1; MaNI-Ax2
Microcystis aeruginosa PCC 9806	Ma98	Ma98-5; Ma98-2; Ma98-2s; Ma98-Ax1; Ma98-Ax2
Nostoc punctiforme PCC 73102	Nosp	Nosp-5; Nosp-2; Nosp-2s; Nosp-Ax
Oscillatoria acuminata PCC 6304	Osa63	Os63-5; Os63-2; Os63-417
Oscillatoria sp. PCC 6506	Os65	Os65-5; Os65-2; Os65-417; Os65-Ax
Prochlorococcus marinus MIT 9202	Pm92	Pm92-5; Pm92-2; Pm92-417; Pm92-Ax
Prochlorococcus marinus MIT 9312	Pm93	Pm93-5; Pm93-2; Pm93-417; Pm93-Ax
Prochlorococcus marinus NATL1A	PmN1	PmN1-5; PmN1-2; PmN1-2s; PmN1-Ax
Pseudanabaena biceps PCC 7429	Psbi	Psbi-5; Psbi-2; Psbi-2s; Psbi-Ax

Organism	abbreviation	Abbreviated protein names
Rivularia sp. PCC 7116	Riv	Riv-5; Riv-2; Riv-417; Riv-Ax1; Riv-Ax2
Synechococcus elongatus PCC 7942	Sn79	Sn79-5; Sn79-2; Sn79-417; Sn79-Ax
Synechococcus ps. JA-2-3B'a(2-13)	SnJA2	SnJA2-5; SnJA2-417
Synechococcus sp. JA-3-3Ab	SnJA3	SnJA3-5; SnJA3-417
Synechococcus sp. BL107	SnBL	SnBL-5; SnBL-2; SnBL-417; SnBL-Ax
Synechococcus sp. CB0205	SnCB	SnCB-5; SnCB-2; SnCB-417; SnCB-Ax
Synechococcus sp. PCC 7335	Sn73	Sn73-5; Sn73-2; Sn73-417; Sn73-Ax1; Sn73-Ax2
Synechococcus sp. RS9916	SnRS	SnRS-5; SnRS-2; SnRS-417; SnRS-Ax
Synechococcus sp. WH 5701	SnWH	SnWH-5; SnWH-2; SnWH-417; SnWH-Ax
Synechocystis sp. PCC 6803	Ss68	Ss68-5; Ss68-2; Ss68-2s; Ss68-Ax
Synechocystis sp. PCC 7509	Ss75	Ss75-5; Ss75-2; Ss75-2s
Thermosynechococcus elongatus BP-1	Te	Te-5; Te-2; Te-2s; Te-Ax
Trichodesmium erythraeum IMS101	Trer	Trer-5; Trer-2; Trer-417; Trer-Ax
Xenococcus sp. PCC 7305	Xn	Xn-5; Xn-2; Xn-2s; Xn-Ax

The phylogenetic tree of cyanobacterial FtsHases correlates highly with the proposed above pattern of their evolution, i.e., one FtsH5 with two Type-B proteins forming a core of FtsHases complemented by one or more proteins from the group of auxiliary FtsHases.

Without a single exception, all proteins denoted as FtsH5 were clearly forming a distinct clade within the tree, Figure VI.2. The seeming exception, the second FtsH5-like protein (Ac54-5s) in *Acaryochloris sp.* CCMEE 5410, was grouped into the type of auxiliary FtsHases, emphasising the existence of a single FtsH5 copy in each cyanobacterium and that accumulation of more than four FtsHases is always on the account of auxiliary FtsHases.

FtsHases categorised into Type-B group, namely FtsH2, FtsH2s and 417FtsHases, formed a large super-clade within the tree, divided nonetheless into two smaller branches. While the first branch was exclusively composed of FtsH2 proteins, the second contained both FtsH2s and 417FtsHases. Grouping the latter two FtsHases into one category suggests orthology with 417FtsHases despite the slight inclination of the similarity score towards FtsH2. For that reason, and in the absence of any experimental

data on their functional role, both groups (FtsH2s & 417FtsH) hereafter will be collectively referred to as FtsH2'. The existence of FtsH2' sub-clade along with that of FtsH2 is in support of the hypothesis that the core of FtsHases in cyanobacteria requires the presence of one FtsH5 and two Type-B FtsHases.

As both sub-clades, FtsH2' & FtsH2 appear to have derived from a common ancestor but the latter consistently exhibit typically higher similarity to the archetypal FtsH5, it may therefore be suggested that the FtsH2' proteins did not evolved directly from FtsH5.

Within the group of FtsH2 proteases there are two particular observations that merit some attention. These proteases in marine *Synechococcus* & *Prochlorococcus* strains form a small separate sub-group whereas the same protein (Glv-2) in *Gloebacter violaceous* PCC7421 appears to segregate even further from the proteins in this group, Figure VI.2. Considering the role of FtsH2 in thylakoid membrane housekeeping, the isolation of Glv-2 seems to correlate with the absence of these membranes from *Gloeobacter* species.

The phylogenetic analysis of FtsHases confirmed that all proteins characterised formerly as auxiliary, including the multiple copies in species with more than four FtsHases, formed a distinct clade within the tree. The only protein that stands out of this group is the NP_925595.1 FtsH in *Gloebacter violaceous* PCC 7421 denoted, solely because of similarity scores, as Glv-Ax2. This protein does not seem to cluster with any other examined FtsH. The long outernodal branch however imply accumulation of multiple indels (insertion-deletions), which is obvious from the alignment file (not presented here). Indeed, Glv-Ax2 comprised of 785 amino acids, is by far the longest sequence amongst all FtsHases examined so far.

In conclusion, it seems that the cyanobacterial FtsHases are grouped into three major lineages, namely: 1) the FtsH5 lineage, represented invariably by a sole copy in each organism that comprises the archetypal form; 2) the super-clade of Type-B FtsHases divided always into two equal parts, FtsH2 and FtsH2', and 3) the lineage of Auxiliary proteins in which all species with four or more FtsHases contain at least one protease in this group.

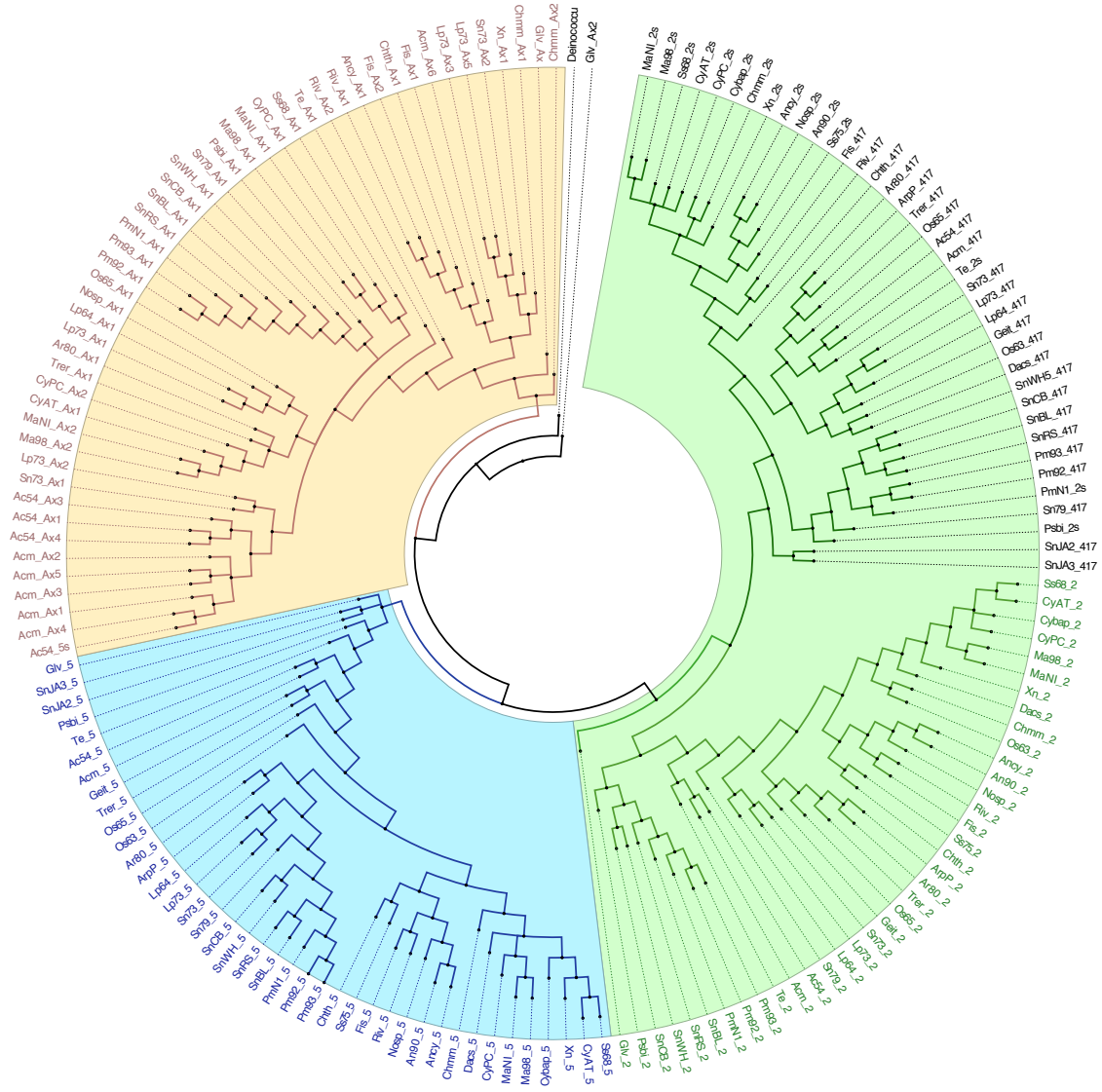

Figure VI.2. Phylogenetic tree of cyanobacterial FtsH proteins. For graphical representation of the phylogenetic tree the programme FigTree (http://tree.bio.ed.ac.uk/software/figtree/) was used.

6. Structural distinctions of cyanobacterial FtsH2 proteins

The universality of FtsH multiplication in plants and cyanobacteria combined with the hetero-oligomeric nature of FtsH-complex along with the multitude of processes associated with these proteases, strongly indicates assignment of different functional roles to each FtsH protein. The protease FtsH2, although dispensable, it is nonetheless of the essence to TM quality regulation mechanism. Involvement of FtsH5 in formation of chloroplast thylakoid membranes does not exclude its involvement in other regulations or functions outside of it. The lethality of *fts*H5 mutation in cyanobacteria is a positive indication for such involvement. Distinctions in functional implications for each FtsH i.e., from PS-II repair cycle of FtsH2, to cell viability of FtsH5, and to complete dispensability of auxiliary FtsHases, must have been inevitably accompanied by a number of changes in their primary structure. It has been reported for instance that a distinct stretch of 81 amino acids in the lumenal loop between the two transmembrane helices distinguishes the FtsH2 proteins of oxygenic phototrophs (Bailey et al., 2002). Yet, Type-A and B FtsHases in *A. thaliana*, despite the overall similarity of their mature forms, differ in their amino-terminal targeting sequences (Rodrigues et al., 20011). Furthermore, the lateral gene transfer from chloroplast to the nucleus during endosymbiosis that has necessitated the return of the translation products to the organelle through its double envelope, is certain to have resulted in important changes as for instance the presence of the appropriate chloroplast targeting sequences e.g. leading peptides for Toc & Tic pathways. Also, the evolution of chloroplast, i.e. transition of the endosymbiont cyanobacterium from a single-celled organism to a semi-autonomous organelle with major changes in spatial organisation of thylakoid membranes into stacked and unstacked parts, is also likely to have imposed additional, key differentiations in plant FtsHases.

Careful examination of the alignment file used for the construction of the phylogenetic tree in Figure VI.2, showed that FtsH2 proteases appear to share an amino-terminal domain of approximately 30 amino acids. Computational analysis of this segment from several cyanobacteria, with tools designed to predict signal peptide sequences (‘SignalP 4.1’ at www.cbs.dtu.dk/services/SignalP; and ‘Phobius’ at www.ebi.ac.uk/Tools), revealed that this particular domain in all arbitrary chosen FtsH2 proteins is a signal peptide sequence. Feature examination of several other FtsH2 proteins as they appear in GenPept Database at NCBI, showed that this particular region was denoted as signal peptide.

To explore whether these residues constitute a domain characterising exclusively this particular type of cyanobacterial proteases, all FtsH2 sequences from hundred and twenty species were aligned (Clustal Omega and MUSCLE) and then analysed with MEME (Multiple Em for Motif Elicitation at www.meme.nbcr.net). The latter program is designed to discover motifs in groups of related proteins. The results of the multiple sequence alignment and the MEME analyses (three sequential, due to limitation in the number of characters simultaneously analysed), confirmed the existence of the amino-terminal domain, universally conserved in all cyanobacterial FtsH2 proteins, Figure VI.3.

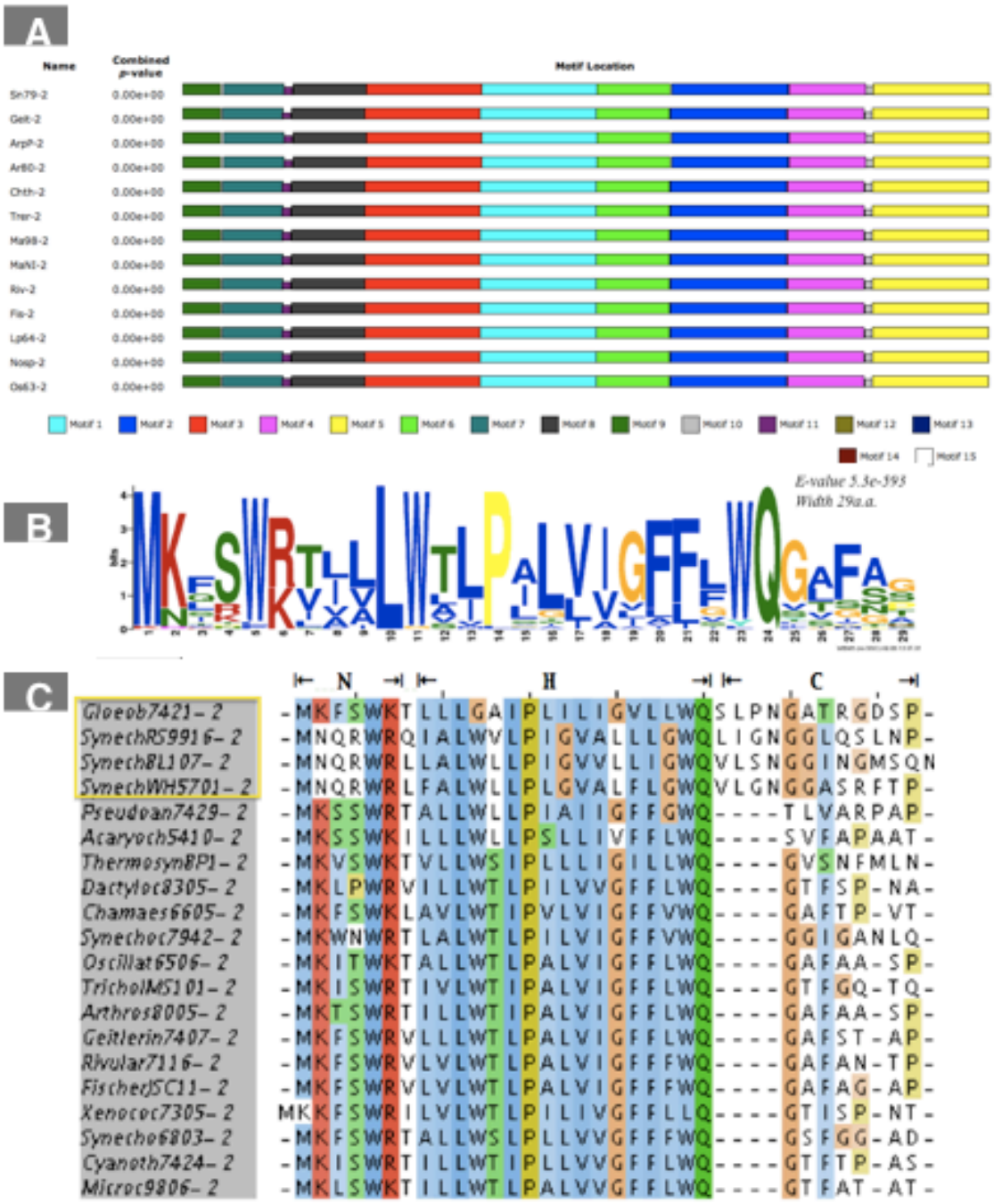

Figure VI.3. Conserved N-terminal domain in cyanobacterial FtsH2 proteins. A) The conserved N-terminal region, Motif-9, as identified by MEME. Small fraction of the overall file presented. B) Sequence logo of the conserved Motif-9. The sequence logo is a graphical representation of aligned sequences where at each position the size of each residue is proportional to its frequency in that position and the total height of all the residues in the position is proportional to the conservation of the position. The logo was computed from the position-specific scoring matrix (PSSM). The colour code is as follows: Blue - most hydrophobic (A,C,F,I,L,V,W,M); Green - Polar non charged, non-aliphatic residues (N,Q,S,T); Magenta - Acidic (D,E); Red - Positively charged (K,R); Pink - H; Orange - G; Yellow - P; Turquoise -Y. C) Part of the multiple sequence alignment file visualised with Jalview. Framed sequences correspond to marine species from the genera of *Synechococcus & Prochlorochoccus* that form a distinct subgroup. Colouring code: Blue -most hydrophobic (A, C, F, I, L, V, W, M); Green - Polar uncharged (N, Q, S, T); Magenta - acidic (negatively charged D,E); Red - basic (positively charged K, R); Pink - Histidine (H); Orange - Glycine (G); Yellow - Proline (P); Turquoise - Tyrosine (Y)

Sequence examination of the conserved region (Figure VI.3, B&C) revealed a typical for signal peptides three-domain structural organisation, composed of hydrophobic region H (approximately the residues 8 to 21) flanked by two polar regions N and C. The same domain appears slightly longer and Glycine (G) enriched in marine *Synechococcus* & *Prochlorochoccus* that as seen in phylogenetic analysis, Figure VI.2, form a small distinct subgroup within FtsH2. This conserved domain (~ 30 amino acids) of cyanobacterial FtsH2 proteins will be henceforth referred to as TP2 (Targeting Peptide 2).

The hydrophobic domain H of TP2 is characterised by the presence of aromatic residues of tryptophan [W], highly conserved at two different positions within this stretch, Figure VI.3.C. Amino acids with large side groups, such as tryptophan and tyrosine [Y] for instance, tend to destabilise α-helical structures. The most distinct however attribute of H, is the presence of two unique amino acids that play crucial structural roles controlling protein's folding pattern. The first amino acid is Proline [P], and it is found approximately in the middle of the hydrophobic region, in almost all examined organisms (absent for some reasons from *Anabaena* species). With a distinctive cyclic structure of the side chain that confers conformational rigidity, proline acts as α-helices and β-sheets disruptor and is commonly found in protein turns e.g. the first residues of an α-helix or in the edges of β-sheet strands. Glycine [G] is the other structurally important amino acid that unlike proline is less conserved at the same position in different cyanobacteria, Figure VI.3.C. Lack of the side chain R in glycine, confers high degree of conformational flexibility that interrupts helical structures. For these reasons, Glycine is often found in sharp turns of peptide backbone, regardless whether its is at the interior or exterior of the protein.

Inasmuch as conservation of TP2 among FtsH2 proteases does not exclude the possibility of TP2 being conserved in other cyanobacterial FtsHases, a series of multiple sequence alignments along with other type of examinations (e.g. BLAST; MEME) were performed to elucidate whether or not this domain is an exclusive characteristic of FtsH2 proteins.

Comparison of FtsH2 with FtsH2' and FtsH-Ax did not only revealed that TP2 is a distinctive feature of FtsH2, but also that the N-terminal part is poorly conserved amongst auxiliary FtsHases whereas almost no conservation pattern exists in FtsH2', Figure VI.4. Use of signal prediction software (SignalP, Phobius) along with the examination of sequence features at NCBI protein database unveiled that many of FtsH-

Ax were not predicted to contain any signal peptides albeit the conservation level higher than in FtsH2'. The level of conservation in N-terminal parts of FtsH-Ax and FtsH2' proteins was confirmed by the number of hits that each type generated in BLAST searches when the first 60 amino acids from many proteins from both types were used as query sequence. While the homology searches with FtsH-Ax produced on average 30-40 hits, exclusively cyanobacteria, the few hits with FtsH2' belonged to the same protein that the query 60 residues were taken from along with a couple of others from very closely related strains. MEME analyses for both types FtsHases correlating highly with the MSAs and the BLAST searches, simply confirmed that TP2 is a distinction characterising FtsH2 proteins.

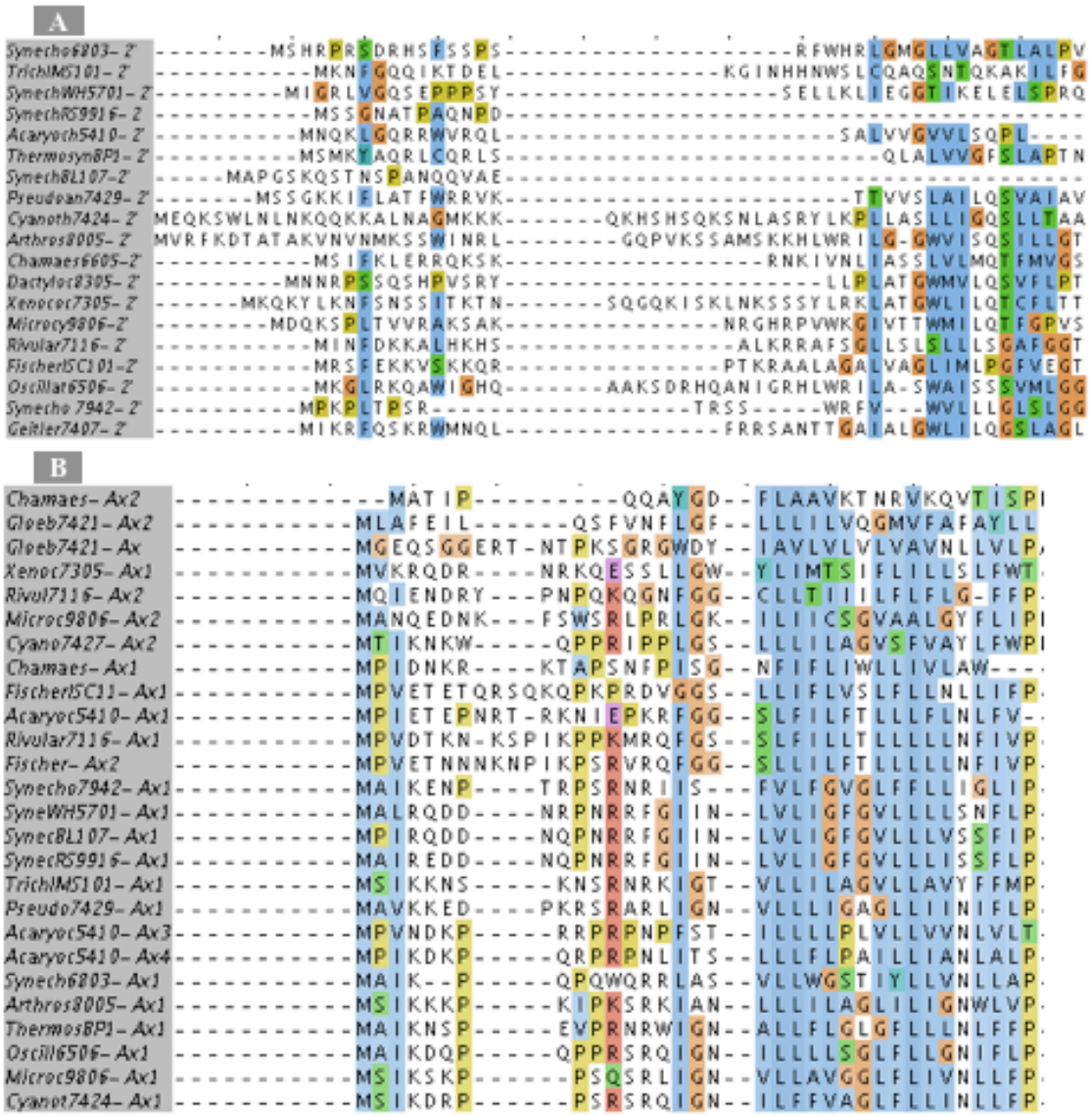

***Figure VI.4*. Multi Sequence Alignments of amino-terminal regions of cyanobacterial FtsHases.** A) FtsH 2‘ & B) FtsH-Aux. The coloring scheme is an in Figure VI.3.

Multi sequence alignments with cyanobacterial FtsH5-ases showed that this group of proteases contain a conserved N-terminal domain of the same length as TP2 but nonetheless different from the latter, Figure VI.5.

The domain is structured in a typical for signal peptides three-parts organisation, consisting of two polar regions (N&C) flanking a larger hydrophobic domain (H). Reference to sequence features at NCBI protein database showed that this part of the protein was annotated as signal peptide. Further analysis with signal prediction software designated this domain as signal peptide as well. Examination however of MSA file and comparison of consensus sequences (Figure VI.3, B and VI.5.B) of TP2 and the signal

peptide of FtsH5 proteins showed that these two conserved domains are different and the latter will be henceforth referred to as TP5.

The degree to which an amino acid is conserved in a specific position in orthologous proteins, strongly depends on the functional or structural role of this residue. In order to understand whether any amino acid within TP2 and TP5 plays such role, the Con-Surf software (http://consurftest.tau.ac.il) designed to identify functionally and structurally important residues in group of closely related proteins, was used. Con-Surf evaluates each amino acid by combination of evolutionary conservation and prediction of solvent accessibility (Glaser *et al.,* 2003). Thus, analysis either of protein sequences or of MSA of the same proteins with Con-Surf (this tool allows such data-input flexibility) unvaryingly produced the same results. In TP2 consensus sequence, the residues M(1); K(2); P(14); Q(24) are predicted to play functional roles (highly conserved and exposed residues), whereas L(10) has structural importance (highly conserved and buried). The numbers in brackets next to each of the residues denote the position of this amino acid in the consensus sequence. The solvent-exposed proline residues [P] are often the sites of protein-protein interaction. In TP5 the amino acids K(3); R(6) and N(7), were predicted to have functional significance and L(10) structural. It appears that any similarity between TP2 and TP5 is restricted mainly to the N region of the signal peptide. Exclusive of L(10) common for both domains, the hydrophobic H region in TP5, in high contrast to that of TP2, has no other residues predicted to be of functional or structural importance. No similarities in the C region are observed between TP2 & TP5 as well.

Simultaneous analysis of FtsH2 and FtsH5 proteins with MEME (Figure VI.5.C), in consistence with MSA and the previous examination of FtsH2 (FigureVI.3.C), confirmed the conservation of two distinct N-terminal regions in FtsH2 and FtsH5, motifs 12 and 10 respectively, FigureVI.5.C.

Distinction between *A. thalina* FtsHases into two different types A & B is based largely onto the translocation mechanisms directing their insertion into the thylakoid membranes. Requirement of proton gradient (ΔpH) across the membrane and the presence of a typical Tat (Twin arginine transport) pathway motif, i.e., two arginine residues [RR] along with a conserved model around [RR] suggested that the delivery and integration of plant Type-B FtsHases (FtsH2 & 8) into the chloroplast TM is accomplished by Tat/ΔpH mechanism. (Rodrigues *et al.,* 2011). In contrast, integration

of Type-A FtsHases (FtsH5 &1) into chloroplast's TM requires ATP hydrolysis and it is achieved by Sec translocation mechanism.

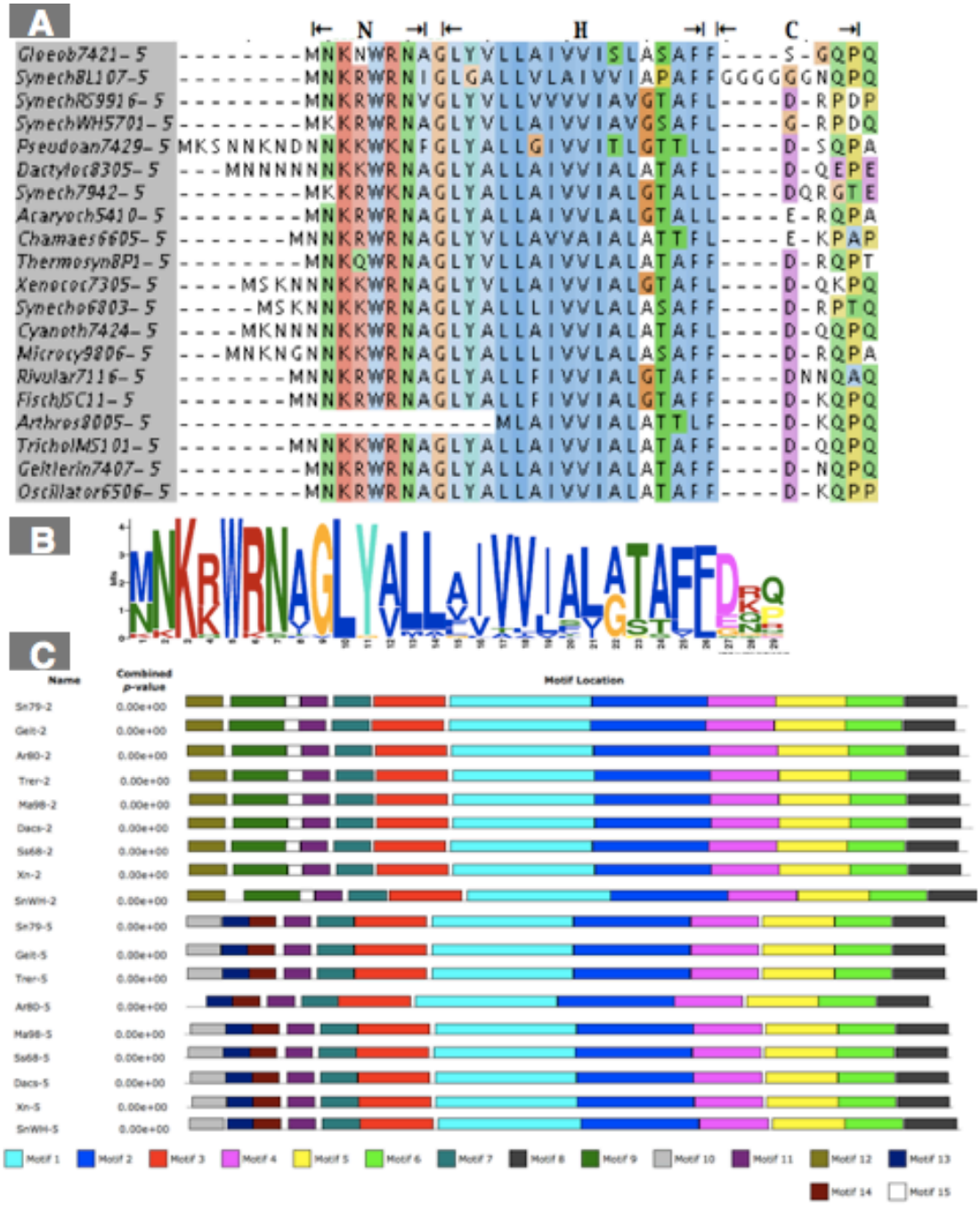

Figure VI.5. Conserved amino terminal domain in cyanobacterial FtsH5 proteins. A) MSA of FtsH5 proteases from cyanobacterial species. A fragment of the whole file is presented. B) Motif 10 - Sequence Logo. C) Conserved motifs in FtsH2 and FtsH5 proteases as established by MEME. The coloring scheme in A & B is as described in Figure VI.3.

Although cyanobacterial FtsH2 proteases do not contain the twin arginine motif, their targeting signals TP2 that seem to contain other important Tat-specificity factors, are profoundly different from TP5. Characteristic features in the entire Tat signal, including regions N, H and C, is what contributes the protein to be recognised by the Tat system and not the presence of arginine motif alone which in certain cases of TM-internal Tat-substrates seems to be completely absent (reviewed in Robinson & Bolhuis 2004).

Lower overall hydrophobicity of H regions in Tat signals compared to Sec, seems to prevent the Tat proteins to be targeted by Sec system. Examination of the hydrophobic domain in both TP2 & TP5 showed that this particular region in TP5, along with the formerly mentioned differences (Proline, Glycine, Tryptophan), is dominated by the amino acids Leucine [L], Valine [V] and Isoleucine [I] which on hydrophobicity scale appear to be the most hydrophobic.

Another important distinguishing characteristic between the two signals lies in the polarity of region C, hence its frequent designation as 'Sec-avoidance signal'. This particular part in Tat-substrates may contain basic or uncharged residues (although less often) but never acidic (Berks *et al.,* 2004). No such residues (Aspartic acid D, Glutamic acid E) were observed in any of the examined TP2 sequences, Figure VI.5., whereas this region in TP5 is characterised by the highly conserved aspartic acid.

Lack of RR motif in TP2 does not exclude the possibility that cyanobacterial FtsH2 proteins are integrated into the thylakoid membranes via ΔpH mechanism. While the presence of twin arginine motif in many Sec-peptides or its mutagenesis in Tat-substrates not resulting in Sec-targeting are all documented facts (reviewed in Robinson & Bolhuis 2004; Pohlschröder *et al.,* 2005) in support of the above statement, the most important evidence for it however derives from the signal-sequences swapping experiments. For a small number of integral thylakoid membrane proteins, it has been shown that their RR motif along with the hydrophobic domain are completely dispensable (Summer *et al.,* 2000; Rodrigues *et al.,* 2011). The plant protein Pftf (plastid fusion/protein translocation factor), is a protease from the AAA-family and closely related to bacterial FtsHases. Although synthesised with a signal peptide bearing typical Tat-recognition features, practically the sole uttermost requirement for integration of Pftf into TM was the presence of pH gradient across the membrane (Summer *et al.,* 2000). Similarly, swapping domains experiments between *A. thaliana* FtsH2 & FtsH5 demonstrated that the primary structure or / and the folded state of the

mature form of FtsH2 that includes the lumenal domain, the second transmembrane anchor, and the large stromaly exposed carboxyl-terminal domain, is incompatible with the Sec translocation mechanism (Rodrigues *et al.,* 2011).

The evident significance of FtsH5 for the viability of cyanobacterial cell along with its integration to the chloroplast thylakoid via Sec-pathway correlate highly with the observations that Sec system, in contrast to Tat, translocates essential proteins and therefore it is of vital importance for growth under all conditions (reviewed in *Robinson & Bolhiius* 20004).

Nevertheless, as the universality of TP2 in cyanobacterial FtsH2 proteins does not exclude the possibility that the same domain may be conserved in FtsHases of other eukaryotic photosynthetic organisms, BLAST searches, sequence comparison (MSA) and MEME analyses between TP2 and the N-terminal segments of FtsHases in eukaryotic organisms were performed.

Homology searches in NCBI Reference Sequence Database having as query the TP2, either from different cyanobacteria, e.g. *Synechococcus* 7942, *Synechocystis* 6803, *Microcystis aeruginosa,* or the consensus sequence of this domain produced virtually the same results. Despite the shortness of the query sequences, only 30 amino acids, all hits belonged to phototrophic organisms, in vast majority cyanobacteria. Notably, only a single hit per organism was recorded, which invariably, as emerged from similarity score tables (Table VI.6) was an FtsH2 protein. Apart from cyanobacteria, a small number of hits from the domains of *Rhodophyta, Cryptophyta, Rhizaria, Alveolata, and Viridiplantae* was also recorded, but it is noteworthy that the hits from the latter two groups were of substantially lower similarity. Yet, examination of the pairwise alignment file for each of these hits revealed that many and long gaps were introduced in case of *Alveolata* and green plants.

Bearing in mind that homology searches with large difference in sequence length between the query and the hits (only 30 residues against entire length proteases of more than 600 amino acids) are prone to produce false hits, especially when the similarity scores are low, further examination of the results was performed. Conducted multiple sequence alignments (with full length proteins as well as with the first 100 amino-terminal amino acids), along with highly correlated MEME analyses, revealed that TP2 is well conserved in Red algae, *Cryptophytes* and *Paulinella chromatophora (Rhizaria*) but not in Green plants and photosynthetic *Alveolata,* Figure VI.6.

As surprising as this finding may be, careful examination of the data and organism characteristics revealed that as far as the structural organisation of thylakoid membranes and the source of FtsH proteases are concerned, all these organisms seem to share some common features.

Red algal chloroplast, albeit the product of primary endosymbiosis with a cyanobacterium-like cell as its plant counterpart, is distinct from the latter because it uses phycobiliproteins as accessory pigments and most importantly contains unstacked thylakoids. Yet, in contrast to green plants with complete horizontal transfer of *fts*H genes to the nucleus of the host cell, FtsH2 in red algae is still chloroplast encoded. The chloroplasts in *Cryptophyta* have derived from a secondary endosymbiosis event with a eukaryotic organism that has been shown to be a red alga (Douglas *et al.,* 2002; Gould *et al.,* 2008). As their predecessor's, chloroplasts of *cryptophytes* utilise phycobiliproteins to harness the sunlight and do not form stacked grana. FtsH2 proteases identified in *Rhodomonas salina* & *Guilardia theta* are also chloroplast encoded. The only hit from *Rhizaria* is the FtsH2 of *Paulinella chromatophora.* The cyanobacterium-derived, semiautonomous photosynthesising organelle of this organisms contain also unstacked thylakoids and the FtsH2, alike red algae and *cryptophytes,* is 'chloroplast' encoded.

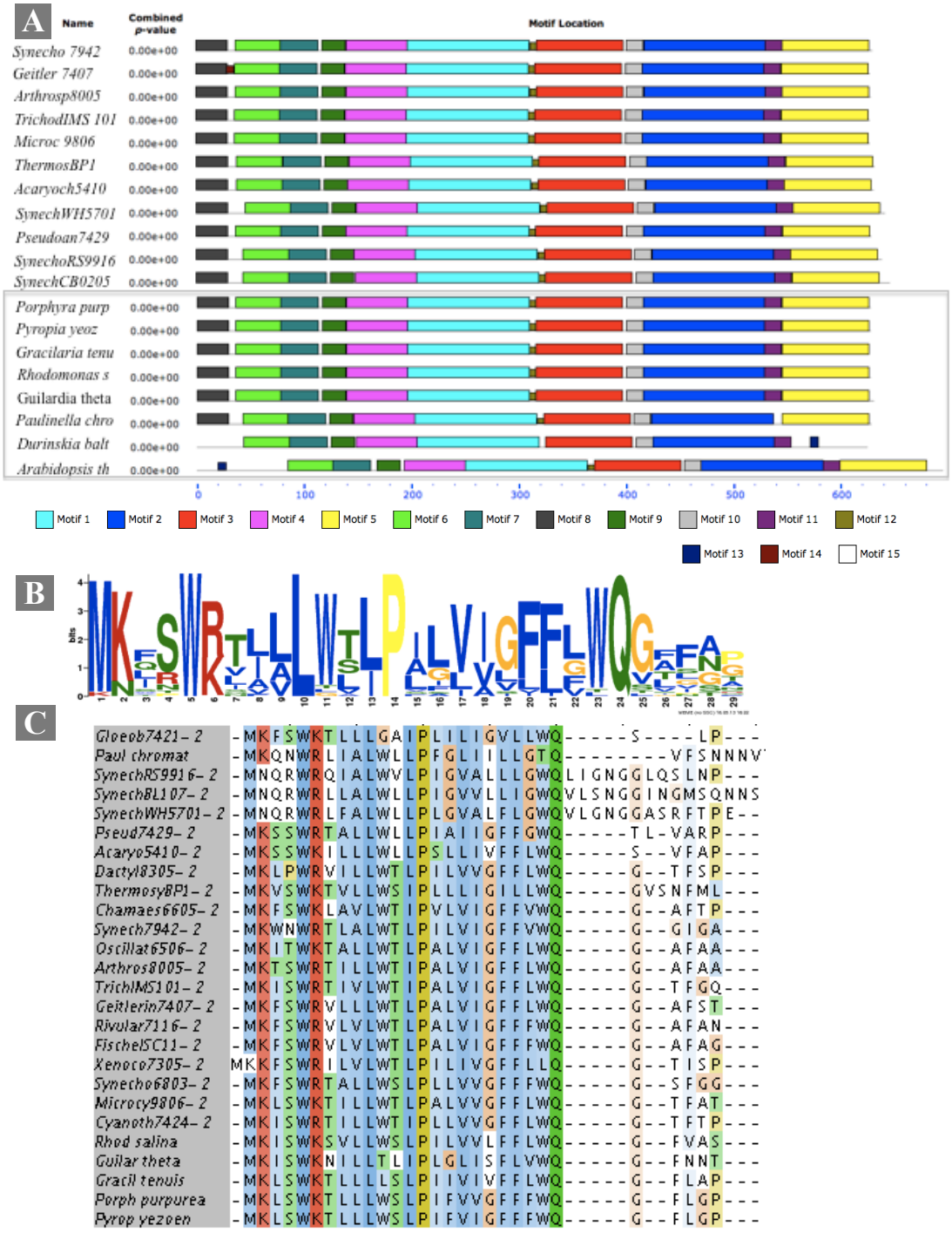

Figure VI.6. Conserved domain TP2 in Cyanobacteria, Red algae & Cryptophytes. **A)** The conserved N-terminal TP2 (Motif-8) identified by MEME. Small fraction of the overall file presented. B) Sequence logo of the conserved Motif-8, calculated from cyanobacteria, red alga and cryptophytes. C) Part of the MSA file visualised with Jalview. Colouring codes in MEME Logo and MSA are the same as in Figure VI.3.

Retention of TP2 in FtsH2 proteases of eukaryotic oxygenic phototrophs with particular type of TM organization suggests the importance of this domain in directing the translocation of these proteins to the right location in the membranes. Given the structural and functional importance of certain amino acids (Proline, Glycine, Tryptophan) at specific positions within TP2, conservation of this domain in evolutionally and taxonomically distant organisms which nonetheless have in principle similar structural organization of photosynthetic membranes, may also indicate that TP2 is important in proper orientation of FtsH2 in TM ensuring thus an integration pattern into the FtsH-complex that is suited for this type of TM organization.

Nevertheless, apart from TP2 that seems to distinguish FtsH2 proteases of oxygenic phototrophs based on the type of TM organisation and the DNA source encoding it, there seems to be a remarkable similarity along the rest of FtsH2 sequence, Figure VI. 6.A. Identified motifs and the order of their occurrence appear to be identical for all cyanobacterial and eukaryotic FtsH2 proteins. The observation acquires a particular weight in case of motifs 6 and 7, Figure VI.6.A., that contain no gaps between them and together have a length of nearly 80 amino acids. Feature examination for most of the proteins at NCBI protein database, along with analysis of these sequences with 'Phobius' (www.ebi.ac.uk/Tools), showed that this particular stretch of ~80 residues lies between the predicted transmembrane segments. Sequence examination disclosed that this particular stretch is identical to previously discovered conserved domain and proposed by Bailey *et al.,* 2002 to be a key-identifier of FtsH-subfamily restricted to oxygenic phototrophs. Splitting however this lumenal domain into two smaller segments, motif 6 & 7 has even greater importance when considering that motif 7, composed of 32 amino acids and located towards the second transmembrane segment, is identical to conserved domain of 31 residues discovered by Bailey *et al.,* 2001. The latter lumenal domain of 31amino acids (Bailey *et al.,* 2001) consists part of a larger domain composed of 81residues, and which was reported in Bailey *et al.,* 2002. The conserved region of 81residues (Bailey *et al.,* 2002) stretches over the largest part of the lumenal loop between the two transmembrane segments while the 31 residues fragment consists the end part of it near the second transmembrane segment.

Examination of MSA file between different types of FtsHases revealed that the 31 residues domain is quite well preserved in FtsH2, FtsH2', and FtsH5 proteases whereas

the segment before it (motif 6, ~50 residues), is only similar in FtsH2 and FtsH2' but distinct from FtsH5 and FtsH-Ax.

Disclosure of TP2 along with the identification of the motif in the lumenal loop, does not only corroborate the discovered earlier domain (Bailey *et al.,* 2001; 2002), but most importantly introduces more details into the overall picture of our understanding on FtsH2 *modus operandi* and conservation pattern of structurally and functionally important domains.

7. Summary & Conclusions

Having the four FtsHases of *Synechococcus* 7942 as the query sequence, the homologous proteins in the rest of the phylum of cyanobacteria were identified. Multiplication of FtsHases in these organisms seems to be universal. The distribution pattern of these proteases, as far as their number in each species is concerned is not even and varied from two to the impressive nine FtsHases in one organism. Having said that however it must be elucidated that the majority of cyanobacteria appeared with four FtsHases. Only two species, two *Synechococcus* ecotypes from the hot springs of Yellowstone national park (USA) contained two FtsHases. The rest of cyanobacteria appeared with either three or five FtsHases, whereas only several species had more than five FtsHases.

It is noteworthy that the number of FtsHases in each genus is not fixed, as for instance some *Synechococcus* species appeared to contain two FtsHases (the two isolates form Yellowstone), four (almost all of them) and five in *Syenechococcus* 7335.

The twelve FtsH proteases from our three model organisms, namely *Synechococcus* 7942, *Synechocystis* 6803, and *T. elongatus* BP1 and the other twelve from *A. thaliana* were compared, and based on similarity scores from the MSA and the existent nomenclature of *A. thaliana* FtsHases, the same names were assigned to orthologous proteins.

All cyanobacterial FtsHases were analyzed, similarity scores from BLAST searches and the multiple sequence alignments were compared and phylogenetic tree was constructed. Taking into consideration the similarity scores from countless homology searches among bacteria it appeared the FtsH5 is the ancestral form whereas FtsH2 is the likeliest first derivative.

FtsHases are not randomly distributed in this phylum as there is a well defined pattern of how these proteases evolve and exist in Cyanobacteria as they seemed to form four major clusters: FtsH5; two clusters of Type-B: FtsH2 & FtsH2'; and FtsH-Aux. Regardless of the total FtsH number in each organism there is always a single copy of the archetypal form, FtsH5. Yet there are always two Type-B FtsHases, one of which is invariably FtsH2. The only exception are the two Yellowstone ecotypes, which nevertheless contain only two FtsHases. In species with more than four FtsHases, every protein beyond the first three, namely, FtsH5 and two Type-B, is always from the group of auxiliary FtsHases which seemed to have evolved in cyanobacteria after the initial primary endosymbiosis event with a eukaryotic cell.

The distribution of FtsHases in the world of living organisms is inconsistent, certainly not universal, and varying significantly not only between the three domains but also within each one separately.

In Archaea, despite the presence of a handful of proteases from the AAA superfamily designated as FtsH, none of them fits clearly into this family of proteases. Particular selective pressure, e.g. membrane composition, structure, and organisation in Archaea may be the reason for absence of FtsH proteases from this domain. Whether this pressure resulted in deletion of *fts*H genes from this domain or they have not been inherited at all from progenotes is difficult to define.

FtsHases are universal in the domain of Bacteria. Their quantitative presence however varies significantly amongst its species. Although in most bacteria FtsHases are present as a single copy, in multitude of others, far too many to be called or seen as exceptions, FtsHases exist in greater numbers. Exclusive of cyanobacteria, multiplication of FtsHases in any taxa (strains, species, genera or families) does not appear to be related to any characteristic that all these taxa have in common and therefore it is likely to be associated with more particular feature(s) in these organisms.

Multiplication of FtsHases is only universal in the domains of Cyanobacteria and Green plants and seems to have evolved in parallel with the evolution of oxygenic photosynthesis.

The horizontal transfer of *fts*H genes from the endosymbiont to the host's nucleus was not ubiquitous in eukaryotes, neither in those with primary chloroplasts (red & brown algae) nor with secondary (*Cryptophyta*, *Euglenozoa*) or tertiary (*Alveolates*). FtsH2 is still being encoded by the chloroplast's genomes of photosynthetic species in the above

taxa and FtsH5 in plastids of some non-photosynthetic organisms that despite loosing the ability to photosynthesize have retained the organelles.

Although the role of FtsH2 proteases in housekeeping of TM seem to be conserved among all oxygenic phototrophs, there seem to be particular differences in the primary structures of these proteases which are not defined by the model of cellular organization, i.e., bacterial & eukaryotic, but by the organizational model of thylakoid membranes (stacked and unstacked) and maybe by the DNA source encoding them. Thus the FtsH2 in cyanobacteria, red algae, *Cryptophyta* and *P. chromatophora* are distinguished from the rest of FtsH2 orthologs by the presence of a highly conserved domain named TP2.

CHAPTER VII

SYNOPSIS & DISCUSSION

Chapter VII

Synopsis & Discussion

1. Synopsis

FtsH proteases and their role in TM quality control mechanism of cyanobacteria were investigated in this project. To acquire a more comprehensive and all-around understanding of these proteases, analysis of their primary structure and exploration of how they are distributed among living organisms were also conducted.

The *fts*H ORFs in our model organisms were identified and then subjected to insertional inactivation with drug resistance cassettes. The phenotype of the produced *Synechococcus* 7942 mutant lacking the FtsH2 protease was analyzed having always as the reference point the phenotype of the wild type cells.

Deletion of FtsH2 in *Synechococcus* 7942 resulted in a retarded growth rate and evident alteration in pigmentation. With 20% less chlorophyll, and nearly the same amount of phycocyanin, mutant cells appeared to be more bluish in contrast to typical blue-green colour of the wild type.

Exposure of both types of cells to high light intensities brought about severe chlorosis in wild type cells, while it had a negligible effect on the mutant. Reflected in numbers, this chlorosis in wild type cells led to massive 65% chlorophyll reduction in just 48 hours and to an immensely intense loss of PC content, 85% in just 24 hours. In high contrast in the mutant cells the chlorophyll content remained largely unchanged as after 78 hours the recorded chlorophyll loss was just 28% whereas the amount of PC was reduced by 50% after 48 hours. As surprising as the finding of chlorosis of wt may be, it nonetheless correlates highly with the proposed role for FtsH proteases in TM repair mechanism and the quantitative assays of PS-II. The green colour of mutant is ascribed to inability of its cells to degrade and repair the damaged centers of PS-II.

Deletion of FtsH2 in *Synechococcus* 7942 had a considerable impact on the photosynthetic apparatus of this organism, at least as far as the PBS and the two photosystems are concerned.

The significant decline of the ratio of relative fluorescence yield F_{PSI} / F_{PSII} from 1.9 (±0.13) in wt to 1.03 (±0.17) in the mutant, suggesting great changes in the

cellular contents of PS-I and PS-II was confirmed by quantitative assays with flash spectroscopy and radioactively labeled Atrazine respectively.

The amount of cellular PS-I was reduced by 20% in the mutant, result that is highly consistent with studies in *Synechocystis* 6803 (Mann *et al.,* 2000). Along with the evident reduction in numbers of PS-I, lack of particular FtsH led to some apparent structural and / or compositional changes in these reaction centres. The blue-shift, 3.0 nm on average, in low temperature emission spectra with excitation wavelength at 435nm, and the increase of chlorophyll molecules per RC from 176 (±10) in wt to 213 (±11) in the mutant are but strong indications of such changes.

Contrary to PS-I, the overall amount of cellular PS-II, as emerged from fluorescence emission spectra, at 77K and RT, appeared to be significantly increased. Despite that, the amount of functional PS-II complexes, as shown by ^{14}C -Atrazine binding assay, was reduced to half, and only these reaction centers, the active ones, appeared to participate in state transitions.

The light harvesting PBS, albeit properly attached to reaction centres in both wt and $FtsH^-$ less mutant, are nonetheless likely to be associated mainly, if not exclusively, with the functional PS-II complexes, and their cellular content in the mutant cells is likely to have been reduced. Degradation of phycobilisomes under Nitrogen-stress conditions in cyanobacteria is an ordered process (Yamanaka, & Glazer. 1980; Collier & Grossman 1992; Reithman *et al.,* 1988), which nevertheless does not appear to require the presence of FtsH2.

The finding that FtsH2 in particular, and no other FtsH, is still encoded by the chloroplast DNA of certain eukaryotic phototrophs but not by the plastids of closely related species that retained the organelle despite loosing the ability to photosynthesize emphasizes the importance of these proteases for the photosynthetic apparatus.

As numerous as the changes to photosynthetic apparatus the deletion of FtsH2 may have caused, the transmission electron microscopy images of wt and FtsH2-less mutant of *Synechocystis* 6803, grown under different light conditions have shown that the absence of these protease resulted in no apparent alterations in distribution patterns of TM. Based on this observation alone, FtsH2 is unlikely to play an important role in biogenesis and spatial organisation of photosynthetic membranes, at least in cyanobacteria. Considering however the roles of its

eukaryotic ortholog, there may be an alternative explanation to its potential involvement in TM biogenesis, and which is considered in the next part of this chapter, Discussion.

With the mutant cells not being affected by chlorosis, despite the intense damage to PS-II under high light; with the overall concentration of PS-II significantly increased but with the number of functional complexes reduced to half, it is evident that lack of FtsH2 in the *Synechococcus* 7942 mutant have greatly impeded the repair mechanism of photosystem-II. The recordings of PS-II activity and of its repair cycle by measurements of oxygen evolution in the presence and absence of lincomycin, substantiate such key-role for FtsH2 proteases in the repair mechanism. Addition of lincomycin to mutant cells has no effect on the course of oxygen evolution, which is rapid decline, identical in both cases, with and without the antibiotic. The activity and the importance of a repair cycle in operation becomes apparent with the addition of lincomycin to wild type cells inasmuch as it leads to a pronounced reduction in rates of oxygen evolution. The impact of FtsH2 on the repair mechanism becomes more explicit when all of the above changes are reflected in numbers. Thus the rate of oxygen evolution in mutant declines by more than 90% in just 25 minutes, with and without the lincomycin. In high contrast, this decline in wild type within the same amount of time is less than 10% when the protein synthesis is unrestricted (absence of lincomycin). The recovery of PS-II activity in cells lacking FtsH2 is practically non-existent.

However, it is worth stressing that the repair mechanism itself appears to be time and stress dependent process, i.e., attainment of maximum efficiency after certain amount of time and inability to maintain it for long period under stress conditions.

The repair cycle of photosystem-II is not an all-weather mechanism, i.e. appears to function with different *modi operandi,* at least under specific light conditions. Thus, under normal conditions where no mobile populations of PS-II can be observed (Sarcina & Mullineaux 2004) but with inactivation in progress, the repair of damaged reaction centres is plausible to take place *in situ.* Exposure of cells to red light on the other hand side triggers mobilisation of certain percentage of PS-II that is believed to facilitate the repair cycle (Sarcina *et al.,* 2003). The contrasting responses of the repair mechanism to different wavelengths are in support for the above proposal. While red and blue lights are equally effective at inducing inactivation of PS-II activity, the former nonetheless triggers an

immediate response of the repair mechanism in contrast to the latter under which the response is considerably slower.

Insertional inactivation of *fts*H2 ORF has apparently changed the dynamics within thylakoid membranes even under normal conditions, inasmuch as certain population of PS-II, as emerges from FRAP analysis, appears to be in a mobile state. Considering the role of FtsH2 in the repair mechanism and its similarity to red pepper's Pftf translocation factor (Hugueney *et al.,* 1995; Chen *et al.,* 2000), it plausible that this diffusing fraction of PS-II in FtsH2-less mutant is composed mainly, if not exclusively, from damaged complexes which in normal cells are immobilized by the protease to undergo *in situ* repair.

While there seems to be little doubt about the significance of FtsH2 in the repair mechanism of PS-II, growth of *Synechococcus* 7942 cells in nitrogen-depleted medium has unveiled yet another possible functional implication of this particular protease, i.e. involvement in degradation of functional reaction centers when abiotic stress conditions require such action. Nonetheless, as loss of functional PS-II complexes occurs in both wt and mutant but to a significantly greater extent in the former (~50% vs ~30%) it seems that the stoichiometry of FtsH-complex in this case is different from that required for PS-II repair.

The distribution of FtsHases in the world of living organisms is inconsistent, certainly not universal, and varying significantly not only between the three domains but also within each one separately.

Archaea seem to contain no FtsH proteins although they are rich in AAA proteases some of which, probably too hastily, were classified as FtsHases. Absence of these proteases from Archaea can be explained by two, principally different conventions. They either have never evolved in these organisms or, given that in the three domain classification of life (Woese 1987; 2000; 2004), Archaea stand between Bacteria and Eukaryotes where FtsHases are ubiquitous, their absence is then may be the result of deletion of these vain genes. Their products are of no service to the membrane regulatory mechanism due to profound differences in membrane's compositional and structural organization. However, if the presence of FtsHases in the world of eukaryotes stems exclusively from bacteria, result of a horizontal gene transfer from the ancestral endosymbiont of mitochondria or / and chloroplast, then indeed the FtsHases may have never involved in Archaea, albeit evolutionary standing between Bacteria & Eukaryotes.

Although FtsHases are universal in the domain of Bacteria, their quantitative presence varies significantly amongst its species. In most organisms these proteases are present as a single copy. Nevertheless in legions of others, far too many to be called or seen as exceptions, FtsHases have multiplied to greater numbers. Exclusive of cyanobacteria, multiplication of FtsHases in any taxa (strains, species, genera or families) does not appear to be related to any characteristic that all these taxa have in common and therefore it is likely to be associated with more particular feature(s) in these organisms.

From the search on FtsH distribution emerged that multiplication of these proteases in Cyanobacteria and Green plants have evolved in parallel with the evolution of oxygenic photosynthesis (more details in Chapter VI). The idea is highly consistent with the current understanding on FtsH involvement in TM housekeeping. Unlike any other bacterial phylum, in cyanobacteria, the FtsH multigene families seem be to be a universal phenomenon and four is the predominant number of these proteases in these organisms.

Based on the accumulated knowledge and the nomenclature in use, cyanobacterial FtsHases were analyzed, classified and then named according to the established phylogenetic relationships. FtsH5 appeared to be the ancestral form. Each cyanobacterium, regardless of the number of FtsHases it possesses, always have a single copy of the archetypal protein (FtsH5) and two Type-B FtsHases one of which is invariably FtsH2. In species with more than four FtsHases, every protein beyond the mandatory three, viz., FtsH5 and two Type-B, is from the group of auxiliary FtsHases. However, evolution of the Auxiliary FtsHases in cyanobacteria looks to have taken place after the initial primary endosymbiosis event with a eukaryotic cell that led to the evolution of chloroplasts.

The lateral transfer of *fts*H genes from the endosymbiont to the host's nucleus was not spread universally among eukaryotes, neither in those with primary chloroplasts (red & brown algae) nor with secondary (*Cryptophyta*, *Euglenozoa*) or tertiary (*Alveolates*). FtsH2 is still being encoded by the chloroplast's genomes of photosynthetic species in the above taxa and FtsH5 in plastids of some non-photosynthetic organisms that despite loosing the ability to photosynthesize have retained the organelles.

Although the key-role of FtsH2 proteases in TM quality control mechanism seem to be universally conserved among all oxygenic phototrophs, there seem to be specific differences in the primary structures of these particular proteases that are not defined by the model of cellular organization, viz., bacterial and eukaryotic, but by the organizational model of thylakoid membranes (stacked and unstacked) and probably by the source of DNA encoding them. Thus the identified TP2 region is a highly conserved N-terminal domain, unique to FtsH2 proteases in cyanobacteria and only in those eukaryotes that still retain the *fts*H2 ORF in chloroplast's genome and have their TM not organized into grana and lamellae.

2. Discussion

To overcome stress conditions or restore any damage, all living organisms have evolved sophisticated and well synchronized protein-quality-control systems with a number of particular proteins engaged in these processes, either as proteases or chaperones or even both. Since the early stages of the research on PS-II turnover, the degradation of photo-inactivated D1 protein was believed to be of proteolytic nature (Ohad *et al., 1985*; Reisman & Ohad 1986), and the initial cleavage was found to take place between the fourth and fifth transmembrane segments of D1 subunit, located on the stromal side of the thylakoid (Greenberg *et al.,* 1987).

Although there seems to be little doubt about the proteolytic nature of this quality-control system, determination of the protein(s) involved in it, and especially those engaged in the early stages of degradation, is still subject of research and debate (reviewed by: Adam & Clarke, 2002; Edelman & Mattoo 2008; Nixon *et al.,* 2005 and 2010). The evidence for one of the prime candidates in this process, DegP2 protein from HtrA serine-proteases family has derived exclusively from *in vitro* studies with chloroplasts (Spetea *et al.,* 1999; 2000; Hauβuhl *et al.*, 2001; Lindahl *et al.,* 1996; 2000). *In vivo* research in cyanobacteria, where all *degP* genes were mutationally inactivated, showed clearly no functional implications in D1 turnover (Silva *et al.,* 2002; Barker *et al.,* 2006). In contrast, *in vivo* studies with FtsHases have repeatedly demonstrated the consequences of FtsH absence to the D1 repair mechanism in both cyanobacteria and eukaryotes (Silva *et al*., 2003; Bailey *et al.,* 2002; Komenda *et al.,* 2006; Kato *et al.,* 2009) As a result it may be hypothesised that the involvement of FtsHases has been universally conserved among oxygenic phototrophs whereas the engagement of DegP proteases has evolved in parallel with the gradual transmutation of endosymbiont

cyanobacterium to the organelle of chloroplast. Regardless however of what one's views upon this matter may be, it is worth stressing that despite the richness of the proteolytical arsenals in both, eukaryotes and cyanobacteria, e.g. 62 proteases in *Synechocystis* 6803 (Sokolenko *et al.,*2002), hardly any research has been done on potential engagement of other proteases in PS-II turnover, either directly as proteases (e.g. as homo or/and part of heterorcomplexes with other proteins) or indirectly (e.g. chaperons etc).

The current picture of our understanding on D1 turnover, albeit grown considerably in details, is still far from conclusive. For these reasons, there is need to discuss a number of certain issues, that have emerged either from the current project or / and the accumulated knowledge on this subject. Thus, bearing in mind the results and the complexity, not only of the repair process but also that of FtsH-complexes (multiplicity, multifunctionality, and holoenzyme's oligomerization), three main areas have emerged that merit more consideration. In particular: a) what other responses or consequences, outside the zone of D1 turnover or in some indirect relationship with it, the deletion of FsH2 has brought forth and which are worth of further investigation; b) FtsHases and the turnover of D1 c) what are the relationships between FtsHases and other proteins in forming a functional complex suited for one or another catalytic reaction and what are the modulators regulating its activity, e.g. how deletion of FtsH2 has affected the formation of an operational FtsH-holoenzyme involved in this process.

The aforementioned subjects were selected for further discussion because it is author's belief that acquisition of more comprehensive understanding in these areas will help to decipher not only the constituent elements of the maintenance mechanism of photosynthetic apparatus but also the interrelationships presiding their regulation and activity. Thus for instance as the degradation of D1 is a processes synchronised with the availability of this subunit (Komenda & Barber 1995; Komenda *et al.,* 2000), then the activity of FtsH-complex is likely to be also regulated. A bare knowledge therefore, on the role of FtsH2, deficient in all-around understanding of the importance of other modulators and FtsHases for example, not only will be insufficient to explain the mechanism, but any further investigations focused exclusively or mainly on FtsH2 will certainly lead to a dead end.

Along with the above mentioned subjects, and as a final part of this thesis, some thoughts on the widely accepted views, conventions and approaches in photoinhibition, will be shared as well.

2.1. Side effects of *ftsH*2 deletion?

Cell division

Disruption of *fts*H2 gene in our model organism has evidently affected the growth rate of the cells (Chapter IV). Deletion of the same gene (*fts*H2*)* in another cyanobacterium, *Synechocystis* 6803, resulted also in reduced growth rates (Silva *et al.,* 2002).

Considering the frequent designation of these proteases as cell division proteins and on the other hand the origin of their name, viz. '*fts*' as filament-forming temperature sensitive due to inability to septate, the effect then on cell's division frequency is to one or another degree anticipated. The mechanism however causing it, remains completely unknown and elusive. Is this the result of a missing element in the process of cell division due to FtsH2 involvement in its synthesis / activation / deactivation or it is because FtsH2 plays a chaperone like role in the formation of an active compound involved in the process? Studies have shown the presence of numerous such proteins (*fts*A, *fts*I, *fts*H, *fts*K, *fts*L, *fts*N, etc) that are required for proper bacterial cell division (Lutkenhaus & Addinall 1997). Although investigation of FtsH2 role in cell division may lie outside the scope of the current project, recording details and gleaning knowledge from these observations may indeed provide important clues if we are to understand better FtsHases and their 'behaviour'.

Mutations of *fts*H in *E. coli* failed to septate and formed elongated filaments. In cyanobacteria however, the FtsH2 less mutant cells despite having slower division rates, did not fail to septate. In numerous observations under the microscope (if the truth to be said, not for this reason though), neither *Synechococcus* 7942 nor *Synechocystis* 6803 FtsH2-less mutant cells appeared to have any visible aberrations from the wild type cells at any stage of their growth. Even the distribution of TM in *Synechocystis* 68903 FtsH2-less mutant, as observed with TE microscope (Chapter V. Figure V.16) has not been affected in any apparent way.

However, if any abnormalities to be observed, e.g. filamentation, they are most likely to occur in a FtsH5-less mutant inasmuch as FtsH5 (to bypass the lethality of this mutation suggestion are made below) is the ancestral form. Observations of this kind will be interesting as they may provide important clues that will help to understand the interaction patterns between FtsH5 and 2, which although seemingly unrelated to quality-control system they nonetheless seem to form the FtsH-complex (*slr*1604 & *slr*0228) involved in D1 turnover of *Synechocystis* 6803 (Barker *et al.,* 2008). Therefore, knowledge on interaction patterns between FtsHases, i..e, optimum pool size and stoichiometry of all subunits (FtsHases and other proteins) involved for the formation of an operational enzyme engaged in particular process, is certainly of great importance if we are to understand the functional implications of these proteases and their regulation mechanism.

Photosystem-I

Two cyanobacteria, *Synechococcus* 7942 & *Synechocystis* 6803 appeared with reduced concentrations of PS-I (Mann *et al.,* 2000, this thesis) as a result of one particular protease (FtsH2) missing from their proteolytic arsenal. The observed reduction in PS-I numbers might be a mere regulation of photosystems (PS-I & PS-II) content as a response of the cell to particular conditions i.e., depleted ability to control PS-II repair mechanism, so PS-I is reduced to maintain an optimum stoichiometry between the photosystems (PS-I/PS-II). Although this explanation seems to be very reasonable, there is however an inclination to believe that this reduction is not due to any such regulation but is the result of FtsH2 activity, more precisely lack of its activity. In other words, the reduction occurred because FtsH2 plays a more important role, e.g. chaperone-like and not only, in the assembly of photosystem-I. The reasons for this proposition lie in the recorded blue-shift in the low temperature emission spectra and in the increased number of chlorophyll molecules per reaction centre (Chapter V), that in turn imply some structural and compositional changes.

With none of the above reasons for PS-I reduction being mutually exclusive, it is nonetheless interesting to test the latter version with further investigation. Because there are numerous possible ways, direct or indirect through which the lack of FtsH2 might have affected the quantity of PS-I as well as the quality state of it, a more methodical approach is certainly required to gain deeper understanding on

this subject and which unfortunately drew little attention from the research community so far.

Thus for instance, using sucrose density centrifugation of fractionated cells and SDS-PAGE analysis, it will be interesting to know whether the mutation, of any cyanobacterial FtsHases will have any impact, complete or partial, on the structural organisation of PS-I centres into trimers.

Decrease of PS-I centers might be the result of reduced quantities of its subunits either due to affected transcription rates of *psa* genes or translation of its mRNAs. The bacterial FtsH has been suggested to be involved in protein expression by affecting RNA synthesis and turnover (Granger *et al.,* 1998; Wang *et al.,* 1998). Furthermore, this 'multitalented' protein (FtsH) is also known to degrade unassembled sigma factors initiating RNA synthesis, i..e, σ^{32} (σ^{H})-factor regulating the expression of ~17 genes in *E. coli* involved in heat-shock response (Tomoyasu *et al.,* 1995). As true quality-control protease, bacterial FtsH has also been found to degrade unassembled SecY subunits of Sec-translocation pathway (Akiyama *et al.,* 1996), that otherwise their accumulation may be harmful for the cell. It appears thus that FtsHases may affect, in one or another way the accumulation of some or all PS-I subunits and it has been shown indeed that lack of FtsH2 in *Synechocystis* 6803 results in reduced presence of PsaD and PsaF subunits (Mann *et al.,* 2000). Based on the fact that accumulation of Photosystem-I subunits is based on the presence of its core PsaA & PsaB proteins (Chitnis *et al.,* 1995), it was assumed (Mann *et al.,* 2000) that the content of core subunits was reduced accordingly as well. However, as the replacement rate of PsaB subunit seems to be the pacemaker reaction in PS-I repair (Sonoike 1996) then it will be interesting to monitor methodically the presence of these core subunits under various conditions and especially under those leading to PS-I photoinhibition, as for instance under low temperatures.

Decline of Photosystem-I centres may also be the result of the missing chaperone-like services of FtsH2 in mutant cells. Bacterial FtsHases have been shown to function as chaperones as well as proteases (Akiyama *et al.,* 1998; Gottesman *et al.,* 1997). Thus, reduced accumulation of PS-I subunits correlating with the quantitative presence of theses reaction centers in FtsH2-less mutant may be in fact the result of such chaperone activities of these proteases.

Other regulations

However, as the bacterial FtsH has also been suggested to participate in degradation of unassembled α-subunits of ATPase complex (Akiyama *et al.,* 1996) along with Rieske Fe/S proteins of cyt-b_6f complex (Ostersetzer & Adam 1997), it would be interesting to investigate the expression (transcription and translation) of these proteins in parallel with those of PS-I centers. Inasmuch as FtsH5 is the ortholog to the bacterial FtsH, potential involvement of FtsH2 in the aforementioned regulations would strongly indicate, that FtsH5 and FtsH2 in cyanobacteria, form a core complex that is modified further by the addition of other subunits and / or modulators suited for the particular reaction they will catalyze.

Antioxidants and Small CAB-like Proteins

As more and more details are emerging around the factors that in one or another way participate or facilitate the assembly of PS-II, or its re-assembly after any damage, an understanding of how these factors themselves are affected in cells with depleted ability to repair the damaged units, will be certainly interesting and may provide important clues on their regulation mechanisms.

It has been shown that oxygenic phototrophs can synthesize a range of antioxidants, e.g. ascorbate, glutathione, tocopherol and others, which are believed to play an important role in protecting the photosynthetic apparatus from oxidative damage caused by ROS or other free radicals (Trebst 2002; Foyer *et al.,* 2006). Yet, the presence of antioxidants has been shown to contribute to faster synthesis of D1 protein and to an enhanced photosynthetic efficiency in *Spirodela* plants (Sopory *et al.,* 1990).

Inasmuch as tocopherol and other antioxidants appear to be an intrinsic part of an overall protective mechanism against photoinhibition, yet more its synthesis is very responsive to the intensity of light (Munne-Bosch & Alegre 2002; Havaux *et al.,* 2005a; Backasch *et al.,* 2005; Kreiger-Liszkay & Trebst 2006), it is important to understand how synthesis of these compounds under various light conditions is affected in cells with a seriously impeded repair mechanism due inactivation of FtsH2. Increased presence of antioxidants in cyanobacterial FtsH2-less cells compared to the wild type may then be an indication not only for their importance in photoprotection, but that the rate actually of damage in the mutant cells would

be even higher. The latter result would suggest even larger importance of FtH2 in photoprotection.

During the repair cycle, Photosystem-II is believed to undergo some disassembly to facilitate the process and certain membrane proteins, such as SCP (Small CAB Proteins) seem to play an important role as carriers of chlorophyll molecules. Although SCP may be part of the anew synthesis of the complex, mutational studies with these proteins (ssl1633 - scpB; ssl2542 - scpC; slr0839 - scpA; ssr1789 - scpE; and ssr2595 - scpD) in *Synechocystis* 6803 have revealed that they are likely to play a role during the repair of Photosystem-II rather in the *de novo* synthesis of the complex (reviewed in Nixon *et al.,* 2010).

Accumulation of SCP in FtsH2-less cells, if larger than in wt, may be an indication that SCP, given that their expression (as *hli:* high light inducible genes) is light regulated, are also the substrate of FtsH-complex. After all, FtsHases are emerging to be an essential element of membrane's proteolytic quality mechanism as they have been shown to participate in the removal of numerous unassembled, misassembled or damaged proteins, notably, not different subunits of the same complex but subunits in different complexes e.g. Rieske F/S of cytochrome-b_6f (Ostersetzer & Adam 1997); *a*-subunits of ATPase complex (Akiyama *et al.,* 1996); SecY subunits of Sec translocator (Akiyama *et al.,* 1996); σ^{32}-factors of RNA polymerase (Tomoyasu *et al.,* 1995). It appears that they are involved in these processes as inducible enzymes, i.e., required and available when their substrate is available, i.e. availability of the substrate regulates the presence of the enzyme that catalyzes its degradation. Bearing in mind the number of quality regulations FtsHases are involved in, and the almost continuous damage of D1 as well, then their constant present in cells as inducible enzymes cease to be a surprise. Furthermore, as the functional significance of each FtsH protein seems to be different (as inferred from the severity degree of their absence in corresponding mutations) it appears that there may be one or just few types of core-FtsH-complexes that by the addition of particular FtsHase(s) or / and other modulators, and in quantities dictated by the specific substrate, the fully operational FtsH holoenzymes are formed.

Phycobilisomes and Thylakoid membranes

Absence of FtsH2 from the cyanobacterial cell, did not appear to affect the mode of attachment of phycobilisomes to the reaction centers. Yet, when the cells were

in great need for nitrogen, degradation of these large peripheral antennas as the source for the missing element, did not involve, at least in any apparent way, this particular FtsH. The results are not surprising and they correlate well with the proposed role for FtsHases as part of the proteolytical machinery controlling the quality state of membrane proteins and phycobilisomes being a peripheral complex. Despite the lack of any direct or indirect indication associating FtsHases with PBS regulation under the examined conditions, the knowledge of quantitative presence of these antennas in FtsH2-less mutants is by no means less appealing. It may not help us to understand the repair mechanism but it will definitely help us to acquire more insights on how these cell respond to these type of conditions.

Under normal light conditions the FtsH2-less cells appeared to contain a larger population of PS-II, although the number of functional RC among them was significantly reduced. Considering that the mutation of FtsH2 has also resulted in some partial mobilization of PS-II, and yet did not prevent state transitions, it would be interesting to understand if the increase of non-functional PS-II centers in mutant cells has somehow affected the diffusion rates of PBS.

In contrast to plants where lack of either FtsH2 or FtsH5 affects the formation of thylakoid membranes (Sakamoto 2003; Sakamoto *et al*., 2003; Yu *et al.,* 2004; Rodrigues *et al.,* 2011), at least in white sections of variegated leaves, lack of FtsH2 from *Synechocystis* 6803 cells, seems to affect neither the formation of photosynthetic membranes nor their distribution as seen with transmission electron microscopy (Chapter V., Figure V.16). A possible explanation for this discrepancy between the functional implications of FtsH2 in plants and cyanobacteria lies in the level of cellular organization, i.e., single-celled of the latter against multicellular of the former. In particular, cells deficient in normal chloroplasts, forming the white sections of the leaves, remain alive by reaping the benefits of being part of a multicellular organism. In contrast to metabolically specialized chloroplasts functioning in a buffered environment provided by the cell and organism as a whole, cyanobacteria as autonomous unicellular organisms are in direct dependence and interaction with the environment. Thus any cyanobacterial cells-organisms that fail to form normal thylakoid membranes, as those of white sections of the leaves, will simply be destined to die, and no such phenotype (deficient in TM) will be observed. For these reasons the possibility of

FtsH2 being involved in the formation of TM in cyanobacteria cannot be completely dismissed. If such functional significance for FtsH2 in cyanobacteria to be discovered, then exploitation of the ability of heterotrophic growth of certain species, e.g. *Synechocystis,* has to be deployed. More details on this and other possible approaches are discussed below.

The lethality of FtsH mutation in Gram-negative bacteria (*E. coli*) has been shown to be the result of unbalanced synthesis of phospholipids and LPS (Ogura *et al.,* 1999). Although this regulation in cyanobacteria is most likely to be associated with FtsH5 rather than FtsH2, as the ancestral form, the multiplicity of these proteases may have resulted in involvement of other FtsHases, as complement to FtsH5, in the process of membrane components regulation. Using chromatography methods, it has been shown that the phospholipid and LPS content in *Synechocystist* 6803 wt cells did not actually differ from that in FtsH2-less mutant (Silva *et al.,* 2003). However, in the same project (Silva *et al.,* 2003) it was also established that deletion of *fts*H2 may have affected the viscosity of TM by increasing the quantity of saturated fatty acids. Occurrence of similar effect in *Synechococcus* 7942 would be expected to hinder the mobility of PS-II even further. Therefore, knowledge of TM composition in *Synechococcus* 7942 is essential inasmuch as this type of effect on its fluidity would suggest that the recorded coefficient of PS-II diffusion (Chapter V) is even greater but it is only being impeded by the thickness of thylakoid membranes.

2.2. FtsHases and D1 repair mechanism

In spite of the ongoing debate about the nature of the enzyme involved in the initial cleavage of D1 subunit as part of its repair process, there seems to be no doubt about the importance of FtsHases in the proteolysis of D1 and in particular about the importance of FtsH2, a role that has been conserved in both cyanobacteria and eukaryotes

Thus species such *Synechosystis* 6803, *Synechococcus* 7942 and *A. thaliana,* that have been deprived of the services of this particular protease through mutations, displayed a significantly reduced amount of activity of the repair mechanism. As a result of these observations, along with other studies such as those showing co-purification of FtsH proteins with PS-II complexes, FtsH2, via deductive reasoning, was associated, and rightly so, with this specific proteolysis. In great

support of this hypothesis are also the findings presented in Chapter VI, showing not only the universal distribution of FtsH2 in cyanobacteria and green plants, but also its presence in the chloroplast genome of several species and most importantly its absence from the plastids of closely related species that retained the organelles despite loosing the ability to photosynthesize (Chapter VI). It is this absence from such species that signifies the importance of FtsH2 in the repair mechanism. Cells dispose themselves of FtsH2 because there is no such process as PS-II repair in these species and FtsH2 is no longer required.

Nevertheless, as significant FtsH2 may appear to be for the repair cycle, its absence from the proteolytic machinery of the cells, cyanobacterial and eukaryotic, did not seem to be sufficient to induce a total shutdown of D1 repair mechanism, thereby implying involvement of other proteins, either FtsHases or not in this process. This important observation, combined with other findings such as co-purifications of certain FtsHases, led to the conclusion that at least two of these metalloproteases are required for the formation of a functional holoenzyme catalyzing the proteolysis of D1. Indeed, the biogenesis of an operational FtsH-complex in *A. thaliana* has been shown to require two types, A & B of FtsHases. Although the enzyme itself may contain two FtsH homologs from each type, FtsH5 & FtsH1 and Fts2 & FtsH8 respectively, only FtsH5 and FtsH2 appear to be of prime importance, whereas the other two, FtsH1 and FtsH8 seem to have rather redundant functions. In *Synechocystis* 6803 as well, these two proteases, FtsH2 and FtsH5, have also been suggested to be engaged in the holoenzyme. Consistent with the above hypothesis are the findings in Chapter VI, on how FtsHases are distributed among cyanobacteria and green plants. Although indirect, they are nonetheless in great support of the fundamental role of FtsH2 in the quality control mechanism. This is so, because absence of *fts*H*2* gene from any cyanobacterium or green plant would require reconsideration of the hypothesis on their importance in the process as well as of the model of this process as being conserved among all oxygen-evolving photosynthesizers.

Despite the piling up evidence about the importance of FtsH2 in degradation of D1, many aspects of this proteolysis, such composition, stoichiometry and regulation of the holoenzyme are still not well understood. Is for instance the recorded decrease of D1 degradation (Silva *et al.,* 2003) due to reduced numbers of functional enzymes (co-regulation issues between subunits) or due to reduced

capacity of the enzyme because of an altered composition? Although deletion of *fts*H2 in both *Synechocystis* 6803 and *Synechococcus* 7942 have seriously impeded the activity of the Repair mechanism, it is however noteworthy that in the latter the consequences of this particular mutation appear to be more dramatic. At high light intensities, in *Synechococcus* 7942, the activity of the Repair mechanism seems to be non-existent as no recovery of PS-II activity can be recorded (Chapter V, Figures V.6; 7; 8). In some contrast, exposure of *Synchocystis* 6803 to high light as well, although undeniably leads to a greatly reduced rate of D1 repair (Figure 2 in Silva *et al.,* 2003), it is nonetheless clear that some repair is still taking place, implying proteolytic operation. This difference in severity of FtsH2 absence on the repair mechanisms between the two cyanobacteria could be ascribed to differences between these two particular species. However, as inactivation of FtsH2 in *Synechocystis* 6803 was achieved by interruption of the *slr*0228 ORF with a 2Kb Ω fragment at a single site of AccI endonuclease, whereas the disruption of its ortholog in *Synechococccus* 7942 was obtained by insertion of the drug resistance cassette into the restriction sites of two enzymes (MscI & BmgBI) that essentially eliminated half of the ORF (Chapter III), including the AAA cassette, then the severity of the latter mutation is not exclusive to be the result of this type of inactivation.

Nonetheless, as the focal point of this discussion is not the interpretation of these differences between the two mutations, but rather the acquisition of a deeper understanding around the FtsH-complex as a functional enzyme, then there are few other things that merit more consideration.

Because in most studies, the cells or the TM and chloroplasts are often exposed to high light intensities to induce photoinhibition and to test thereby the repair mechanism under such conditions, drawing conclusions from such approaches alone, it is as though assuming the existence of a sole and unique *modus operandi* for the Repair Mechanism under all sort of conditions. Therefore any studies designed to understand photoinhibition as well as the Repair mechanism should include experimentation under various light conditions; low and moderate lights, along with photoinhibitory intensities. Statistically approved differences in the rate of PS-II damage under for example low and high lights, in FtsH2-less mutant alone, could result in interesting conclusions. A severely retarded repair mechanism under high light compared to a lower effect under low light might

imply different overall modes of action of the repair mechanism. Thus for instance, action of a FtsH2 homo-oligomeric complex (or predominantly composed of FtsH2) alongside a FtsH5/FtsH2 hetero-oligomeric complex under extreme circumstances would be a possibility that can explain the recorded differences. Such complexes have been speculated to exist in *A. thaliana* (Sakamoto 2003; Sakamoto *et al.,* 2003).

It has been proposed earlier on in this thesis (Chapter V) that the repair cycle of PS-II is not an all-weather mechanism, at least as far as the location of this process is concerned inasmuch as the quality of light, along with the presence / absence of FtsH2, seemed to have a profound effect of PS-II mobilization. Red light alone appear to trigger mobilization of photosystem-II complexes that is believed to facilitate the repair cycle (Sarcina *et al.,* 2006). FtsH2 on the other hand side seem to be involved in the immobilization of certain PS-II centers (believed to be the photo-damaged ones, Chapter V). Under blue light, where the entire population of PS-II is immobile (Sarcina & Mullineaux 2004), the response of the repair mechanism to the ongoing photo-inactivation is slow, probably due to adoption of a different approach to counteract the problem (exchange of D1:1 with D1:2). Therefore, it will be interesting to see how FtsHases are expressed (transcripts and proteins) under these conditions. This type of data may provide important insights not only on FtsH regulation mechanism but also on that of the repair mechanism. Thus for instance, similar patterns of FtsH expression, combined with the rapid expression of *psb*AII/III genes (Tsinoremas *et al.,* 1994) in wild type alone, would imply that the loss of PS-II activity is compensated (to an acceptable by the cell degree) by the exchange between the two types of D1 subunits.

Despite the well documented effect of *fts*H2 deletion on the repair mechanism, it is nevertheless still unclear how this mutation affects the process. Operation of the repair mechanism based exclusively on FtsH2 homo-oligomeric complexes is rather unlikely, because mutations of this gene would certainly lead to a complete shut down of PS-II repair. If however these homocomplexes exist, they are likely to be backing for the main complex under specific demands. So it appears that FtsH holoenzymes are composed of at least of two FtsHases and the studies so far substantiate this conclusion (Boehm *et al.,* 2012). However how the lack of one subunit affects the function of the holoenzyme is yet to be understood. In

particular, has the absence of FtsH2 resulted in: a) reduced number of enzymes because expressions of FtsH2 and FtsH5 are co-regulated, or b) reduced performance / functional capacity of the enzyme due to its altered stoichiometry? Although in both cases the composition of the enzyme is expected to deviate from the normal, (regardless of wether the missing subunit will be substituted or not or how), understanding what exactly hinders the degradation of D1 will certainly help to understand the regulation mechanism, the mode of function and the role of other FtsHases. Thus for instance if the declined rate of D1 degradation will be proved to be due to insufficient 'workforce' (number of FtsH-holoenzymes), then important inferences can be made in relation to enzyme's regulation. The expression of FtsH subunits (either transcriptional or post-transcriptional) is co-regulated and dictates the accumulation of adequate number of functional holoenzymes. In turn, this co-regulated expression FtsHases implies that they are not, at least directly, regulated by the state of PS-II and therefore there seem be other factors that control their expression. Indeed, Tfh1 (thylakoid formation) has been shown to be a prerequisite for expression of FtsHases in chloroplasts of *A. thaliana* (Zhang *et al.,* 2009). This outcome seems to be consistent with the overall importance of FtsHases. Not being controlled by the state of PS-II, damage of which is taking place almost continually (*in vivo* supposed to cease only at nights), suggests that the presence of these enzymes is not confined to PS-II only but is required for other important regulations, e.g. thylakoid membrane biogenesis, etc. Quantitative assays of FtsH5 expression in FtsH2-less mutant compared to those in wild type will provide such answers. Yet, if there is still a recordable activity of the repair mechanism in the mutant cells, especially under photoinhibitory conditions, then it may be said that FtsH2 is to certain degree replaceable, either by the other core subunit (FtsH5) or by any other FtsH or other protein. In green plants it has been shown that co-regulated expression at protein level is indeed the mechanism controlling the formation of FtsH-holoenzyme (Sakamoto 2003; Sakamoto *et al.,* 2003). If however the expression of FtsH5 remains unchanged, then it may be inferred that these two main subunits are expressed independently one from another. Further conclusions will certainly depend upon other data such as expression of other FtsHases along with co-purification with FtsH5 etc.

The main subject of this chapter is neither interpretation nor speculation on every possible outcome. To the contrary, there is a single and definitive purpose; focus

the attention of the future research on those particular issues that seem to be the missing pieces of the jigsaw puzzle called repair mechanism and photoprotection. Thus, bearing in mind all of the aforementioned points and questions, it appears that if we are to step closer to a deeper understanding of these intricate processes, then embracing new approaches look like the next step forward. Most of the research to the present day has been focusing on recording the 'fate' (synthesis and degradation) of D1 subunit in FtsH2 mutants, and that, under limited set of condition and paying little, if any, attention to the rest of FtsHases. Although our understanding on the functional roles of other FtsHases has been impeded by the importance they seem to have for the viability of the cells, it is nonetheless also true that our studies on these proteins have not been as methodical.

Considering that FtsHases are likely to have different functional role(s) under specific environmental conditions, (Sakamoto 2003), as well as that cyanobacteria contain two types of D1 subunits (Campbell *et al.,* 1995; Golden *et al.,* 1986) that are transiently interchanged under specific stress conditions (Öquist *et al.,* 1995), then parallel investigation of all FtsHases and D1 proteins, under specific sets of light (intensity and quality) and nutrient availability, appears as the next inevitable step to investigate the repair mechanism. Continuing focusing only on one protease (FtsH2) and its impact on one protein (D1) and that only on its one form D1:1, although may produce some more insights, it is unlikely to lead to a comprehensive understanding of this process. In contrast, an all-around understanding may only be the result of studying all parties involved and interconnecting the produced data.

The first step and the less expensive one as well, is to study further the distribution, not only of FtsHases but of the other candidates involved in D1 proteolysis, i.e., DegP protease. Similar data are essential to be obtained for other proteins that seem to modulate or interfere with the activity of FtsH-holoenzymes such as HflK/C complex or R-3-hydroxy- acyl-ACP dehydrase (Ogura *et al.* 1999); Tfh1 (Zhang *et al.,* 2009).

Recording the distribution of FtsHases and other associated proteins among living organisms is far from a mere encyclopedic knowledge. The possibility to be accepted as such it is only if it will remain unassociated with the overall knowledge from experimental data about regulation, functional model and processes these proteases are involved in. Although utilization of such combined

data, cannot provide unambiguous evidence for the role of FtsHases in particular processes, it can certainly exclude unambiguously the possibility of some proteins engaged in specific process and most importantly it can provide the solid ground for other hypotheses and yet to produce important insights on regulation mechanisms and functional implications of other FtsHases.

Thus far, the significance of FtsH2 in TM quality control mechanism has been signified as by their presence in chloroplasts of certain photosynthetic eukaryotes, as by their absence from the plastids of other related non-photosynthetic species. More important clues are likely to be discovered upon sequencing completion of their multiple DNA sources. It will be interesting for example to see how FtsHases and other proteins mentioned above are distributed in cyanelles of *Glaucophyta* that are believed to be closer to the original endosymbiont than any other known organelle or plastid.

To complete the picture on FtsHases distribution and above all to consolidate the hypothesis of FtsH2 as the key protease in PS-II repair cycle, is to investigate the presence of FtsH and DegP proteases in parasitic plants. Higher plants have been shown to contain multiple FtsHases many of which have evolved in parallel with the evolution of photosynthesis. The knowledge of what proteases these plants that lost their ability to photosynthesize have kept and what they disposed of, will certainly lead to important insights. As far as the repair mechanism is concerned, absence of FtsH2 in particular (maybe along with the presence of DegP) will strengthen enormously the hypothesis of this protease as the key factor in TM quality control mechanism. Below is but a short list (in no particular order) of such plants, most of which are being currently sequenced: *Cuscuta europaea* (the greater dodder)*; Orobanche purpurea* - (broom-rape)*; Rafflesia* (~28 species)*; Hydnora* (5 species); *Prosopanche* (2 species); *Monotropa uniflora* (ghost plant, Indian pipe); *Sarcodes sanguinea* (snow plant/flower); *Rhizantella gardners* (western underground orchid); *Knottier nidus-avis* (Bird's nest orchid); *Allotropa virga* (sugar-stick, Barber's pole) etc.

However, what is going to constitute a true quantum leap for the level of our comprehension of PS-II repair is the parallel investigation of all four FtsHases; PS-II complexes & D1 protein along with the activity and mobilization of PS-II under specific conditions of light and nutrient availability. At this point it may be useful to elucidate that parallel investigation by no means refer to a simultaneous

acquisition of all data but it is rather the knowledge of how all of the above mentioned elements and functions are regulated under particular conditions. It will be interesting for example to see how growth of cells under high light has affected not only the activity of PS-II and its repair mechanism but also the real time dynamics of the complexes, the expression and accumulation of all FtsHases and the D1 protein (including fragments, precursor forms etc), the stoichiometry of monomeric and dimeric complexes, accumulation of RC47 complexes (monomeric PS-II lacking CP43), and others. Such knowledge when compared for different set of conditions is what will provide the answers to many questions that remain unknown. Are for example FtsHases involved in degradation of functional photosystem-II under Nitrogen stress and what is the implication of FtsH2 or / and other FtsHases in this process.

Therefore, it is important to acquire data for a number of different conditions, comparison of which will lead to a knowledgeable understanding. Inasmuch as light is part of the process, it will be beneficial to conduct the research under different intensities, i.e. low, moderate and high (photoinhibitory) as well as different spectra e.g. red, blue, etc. Yet, as there seems to be a novel implication for FtsHases, viz., degradation of functional PS-II in quest for nitrogen under limited availability of this element, then understanding the regulation of D1, and of FtsHases under these circumstances, along with how this affects the photochemistry, activity (reflected as oxygen evolution) and mobilization of the PS-II complexes will surely give rise to valuable information.

As far as the knowledge around FtsHases is concerned, it should not be restricted exclusively to FtsH2. Although it is true that significance of two FtsHases has hampered the progress of our understanding on their potential functional involvement, it is also true that in the conducted until now research in FtsH2-less mutants, little attention is payed on how the rest of these Ftshases are expressed, at both mRNA and protein level, or how they co-purify not only in wild type but in the mutant as well. This type of information is certainly not less valuable and therefore should not be ignored in future projects.

In *Synechococcus* 7942, the mutation of *fts*H2 resulted in profound reduction of functional PS-II, whereas the overall population of these reaction centers appeared significantly increased. Thus the understanding of how this mutation has affected the synthesis-degradation of D1 along with monomerisation / dimerisation of

whole complexes and where the increase of the overall population of PS-II could be ascribed to, inactive or damaged centers will be immensely helpful.

The knowledge on PS-II activity (as recorded with measurements of oxygen evolution) under particular light conditions led to some significant conclusions. However additional knowledge of how photosystems-II respond to photoinhibitory conditions when the cells have been previously acclimated to conditions such as high light, or red / blue light will be very useful as well. Similarly, it is also important to understand if or how growth of FtsH2-less mutant cells under low, moderate and high lights, prior to FRAP analyses, has affected the population of mobilized PS-II. For example, as it is known that the growth under high light does not induces chlorosis to the mutant cells as it does to wild type, and retention of the green colour is attributed to inability to repair PS-II, and yet that FtsH2 has been associated with the immobilization of damaged reaction centers, then growth of cells under various light fluences may eventually result in increased number of mobilized populations of PS-II when measured with LSCM in FRAP analyses.

2.3. FtsHases: Regulations, Role allocation and Structural features

A large part of our knowledge on FtsHases comes from *in vitro* studies in *A. thaliana* and *E. coli.* On the other hand, our understanding of these proteases in cyanobacteria, derived from both *in vivo* as well as *in vitro* studies, is based mainly, if not exclusively, upon observations on one particular homolog, FtsH2, although they (cyanobacteria) are known to contain several different copies, most often four. At this point however, it is fair to say that there are particular reasons that impeded the progress of our understanding about the other FtsH proteases.

The first such reason is the significance that two of these proteases, FtsH5 and FtsH2' appeared to have for the viability and homeostasis of cyanobacterial cells. Indeed any attempt to inactivate these two genes, *fts*H5 and *fts*H2', in either *Synechococcus* 7942 (this thesis) or in *Synechocystis* 6803 (Mann *et al.,* 2000) met with not success. Given that the same protocol and procedures resulted in successful mutations of *fts*H2, it appeared that the two particular proteins are of prime importance for both cyanobacterial species. It is however noteworthy that this particular problem is aggravated in cyanobacteria by the fact that they, unlike

green plants, are single-celled organisms (the differences how these particular mutations affect unicellular and multicellular organisms is discussed above).

The second reason however, contrary to the first, is the seeming insignificance that the fourth homolog (FtsH-Aux) appeared to have, since its mutation produced no obvious phenotype (Mann *et al.,* 2000). It is probably due to this functional redundancy in particular, that no further attention was paid to this FtsH. Nevertheless the 'stubborn' presence of this gene in most of cyanobacteria is very unlikely to be a mere coincidence, especially when considering the disposal of *fts*H2 from species that lost the ability to photosynthesize but retained the plastids, remnants of photosynthetic organelles (Chapter VI). Lack of an obvious phenotype, by no means implies that there is no phenotype. The fact that we do not see it, is because, we apparently do not know what to look for. Deletions of *fts*H1 and *fts*H8 in *A. thaliana*, alike *fts*H-Aux in cyanobacteria, produced no obvious phenotypes initially, but because further investigations were carried out it appeared that they participate in the formation of the holoenzyme and can compensate for the loss of FtsH5 and FtsH2 respectively. It is this particular function, compensating the loss of the prime FtsHases that led to the 'threshold hypothesis' explaining leaf variegation in VAR1 and VAR2 mutants.

Regulations

Certain similarities in the formation of an operational FtsH holoenzyme involved in TM housekeeping, suggest that the model at its base may be conserved in cyanobacteria and plants. The biogenesis of the enzyme in both groups, has been shown that requires the presence of two types of FtsHases, A & B, and these two types are ubiquitous among cyanobacteria as well as plants.

To test this hypothesis further and most importantly to bypass the lethality of *fts*H5 *and fts*H2' in cyanobacteria that in turn will allow us to investigate their functional implications, double mutations, i.e., deletion of one FtsH along with over-expression of other, might be proved worthwhile. As important as the proof for a conserved model may be, finding a way to investigate the role of other FtsHases and their regulation is at least as significant. As discussed above, deletion of one subunit only (FtsH2) was not sufficient to allow us understand how each subunit (FtsH5 and FtsH2) contributes to the formation of the holoenzyme. Thus, over-expressions of certain FtsHases, in the mutant background of others, e.g. over-expression of FtsH-Aux in FtsH5 / FtsH2-less

mutant, or over-expression of FtsH2 in FtsH5 / FtsH2' may provide the necessary insights that will help us to formulate a model on how these proteases are regulated to form the FtsH-complex. Co-regulated expression (either at transcripts or protein level) of FtsH5 & FtsH2 will suggest that the impeded rate of D1 degradation during PS-II repair is chiefly because there is insufficient number of FtsH-complexes. In addition to that, if this approach of double mutations (over-expression and deletion) is practically applicable it will also allow us to answer some other important questions as for instance, what are the cause of lethality in each mutation of FtsH5 and FtsH2', or what other reactions these two proteases are catalyzing (degradation of SecA subunits, etc). More details on allocating more particular roles for each FtsH are discussed below.

Along with the mechanism regulating the formation of functional FtsH-complex there is still lack of sufficient knowledge on what regulates the activity of the holoenzyme. The presence alone of an enzyme does not necessarily guarantee that the reaction it catalyzes will occur. Enzymes can be activated or deactivated in many ways. In case of FtsH proteases, especially the cyanobacterial complex, the mechanism regulating its activity is not well understood. What is it that determines the engagement of the enzyme in a particular reaction, being that degradation of damaged D1, or SecY subunit, Fe/S-Rieske protein, etc. As these proteolytic degradations are performed under specific conditions and are highly selective, there must be something that differentiates the substrates from functional complexes. Is it the state of the substrate as the mere factor that determines the activity of the enzyme or there are other substrate specific or / and other modulators that initiate the required degradation.

If the holoenzyme involved in D1 turnover, requires a particular state of the substrate (damaged PS-II complex) to catalyze the degradation then it means that it is constantly (or almost so) active, and diffusing along the TM to repair the damaged photosystems. This is rather a random action as it requires continuous diffusion of the enzyme and to be only activated / start catalysis when and where it finds its substrate. For these reasons we believe that this mode is less likely to be the regulating mechanism.

Existence of other proteins or modulators, substrate specific, that guide the enzyme and regulate its activity is a most favorable alternative explanation. In this case, the overall state of the substrate is then what actually allows the physical

engagement with the enzyme. In *E. coli* for example the complex HflK/C has been shown to regulate (inhibit) the activity of the bacterial FtsH complex (Kihara *et al.,* 1996 and 1998). Although a preliminary homology search for this complex in cyanobacteria showed no significant similarities, it cannot exclude the possibility for their existence. After all HflK/C complex is found to inhibit the FtsH activity in degrading unassembled SecY subunits (Kihara *et al.,* 1997). Consistent with the hypothesis of modulators regulating the activity of the holoenzyme is also the fact that proteolysis and the anew synthesis of D1 in cyanobacteria are well synchronized processes (Komenda & Barber 1995; Komenda *et al.,* 2000). Furthermore, the capacity of the Repair cycle appears also to be contingent upon the availability of chlorophyll and ATP (reviewed by Nixon *et al.,* 2010).

Role allocation

Since FtsHases exist as multigene families, oligomerize in order to catalyze reactions, and are multifunctional then it appears that each FtsH have specific role(s) in the processes it is involved in. Highly consistent with this idea is that FtsHases are found to respond differentially to changes in environmental conditions (Sakamoto *et al.,* 2003; Sakamoto 2003).Yet, it has also been recorded that the variegation in VAR5 & VAR2 mutations in *A. thaliana* depends on environmental conditions.

The lethality associated with the deletion of FtsH5 & FtsH2' in cyanobacteria by no means implies that these two particular proteins are involved in the same vital process. To the contrary, the causes of lethality in each case are expected to be different, i.e., involvement in essential for the cell processes but of different nature. This knowledge remained somehow elusive for the reasons discussed in details above. Along with proposed method of over-expression & deletion to investigate the roles of other FtsHases, there alternative approaches that may be as useful.

The detrimental effect of FtsH deletion in *E. coli* is caused by imbalanced synthesis of LPS and phospholipids (Ogura *et al.,* 1999). However, it has been established that inactivation of the gene encoding R-3-hydroxy-acyl-ACP dehydrase, and which is an essential element in the chain of phospholipids biosynthesis, can actually suppress the effect of FtsH mutation. This suppressor mutation, *sfhC*21, was found to be an allele of *fabZ* gene that encodes the R-3-

hydroxy-acyl-ACP dehydrase and which according to a preliminary homology (query sequence's NSBI accession: NP_285874.1) search appears to have homologous proteins in cyanobacteria.

Bearing in mind that FtsH5 is the ancestral form, it is likely that it will be involved in balanced synthesis of LPS and lipids in cyanobacteria. Based on these observations it will be interesting to see how deletion of *ftsH5* and *ftsH2'* in the background of *sfh* mutation will affect the viability of cells. Suppression of the lethality in FtsH5 mutation, especially if combined with lack of such effect in case of FtsH2', will be a strong indication that: a) FtsH5 and FtsH2' are involved in different processes and b) conserved role for FtsH5, as the ancestral form, in balanced synthesis of LPS and lipids.

An alternative method to understand the functional roles of other FtsHases (and not only) emerges with the introduction of copper-regulated promoter to cyanobacterial cell (Gao & Xu 2009). Thus, the effect of deleting the particular FtsHases from the genomic DNA can be bypassed by the introduction of the same genes into the cells but in a plasmid DNA, expression of which is regulated by the availability of copper in the growth medium. This method, if applicable with FtsHases, can profoundly change our understanding of these proteases.

Studies on FtsH distribution among cyanobacteria (Chapter VI) have shown that the only cyanobacterial species that does not contain FtsH2' is *Gloeobacter violaceus PCC 7421* (Chapter VI). Considering the importance of these protease for the cyanobacterial cell and on the other hand that *Gloeobacter* are the only cyanobacterial organisms that do not contain thylakoid membranes, it might be that the functional importance of FtsH2' arises exactly from its implication in the biogenesis of TM. Utilization of the Cu-induced expression of proteins in cyanobacteria may then help to allocate particular functional roles to all FtsHases

No functional implication were assigned thus far to FtsH-Aux, because as mentioned earlier on, deletion of the genes encoding them did not result in any particular phenotype different from the wild type. Nevertheless, as *A. thaliana* FtsH1 has been suggested that it may be involved in phytochrome-A signal transduction pathway (Tepperman *et al.,* 2001), and given the similarities between FtsH1 and FtsH-Aux (Chapter VI) then it may be interesting to see if the latter protease is somehow involved in the process. If FtsH-Aux is indeed involved in

phytochrome regulation it will be very interesting to see if or how its deletion will affect the mobility of PS-II. Will the red light-effect on PS-II mobilization be preserved in FtsH-Aux less mutants or not?

Structural features

Undoubtedly, the knowledge of functional implications of each FtsHase will improve tremendously our understanding of these proteases (functions, regulation mechanisms, etc). However if we are to understand what are the features in these proteins that the functional differences stem from, i.e., what are those characteristics in the primary structure that make each FtsHase suitable for different reactions, then we need to map those sequence differences in more details.

It has been shown for example that a large lumenal loop between the two transmembrane segments, composed of 81 amino acids, is a feature distinguishing the FtsH proteins in oxygenic phototrophs from the bacterial ones (Bailey *et al.,* 2002). In this thesis we have shown that an N-terminal TP2 domain characterizes only those FtsH2 proteases that are found in oxygenic phototrophs with their TM not organized into stacked and unstacked parts excluding thus the FtsH2 in higher plants. Nevertheless, it is possible that along with the above two segments (81 a.a. lumenal domain and TP2) other fragments in the primary structures of FtsHases may constitute such characteristic features that distinguish each type from another.

To understand the importance of these domains, e.g., implications in the formation of functional FtsH-complex, further experimental work is required. It has been proposed that the N-terminal part of FtsHases is involved in oligomerization and transmembrane organization of the enzyme (Akiyama & Ito 2000). Based on sequence analysis data, it has also been proposed (Rodrigues *et al.,* 2011) that Type-B FtsHases (FtsH2 & 8) in *A. thaliana* undergo maturation process during which a part of the N-terminal domain of the proteins that includes the first transmembrane segment is cleaved off. The mature forms of FtsH2 & 8 consist then of a single transmembrane segment flanked towards the lumen by a small N-terminal domain and on the stromal side by a large carboxyl-terminus which contains the catalytic site of the protease.

Considering the above suggestions along with the high degree of TP2 conservation among cyanobacteria and particular photosynthetic eukaryotes

(Chapter VI), comprehension of the essential elements in the mechanism regulating the formation of an operational enzyme, will require mutagenesis studies. To understand the functional implications of TP2 as a whole, as well as some of its highly conserved residues, such as that of Proline, either in targeting the protease to TM or in the formation of the enzyme, both domain deletions / swapping and point mutations will be critical. Point mutations, would rather be anticipated to have little, if any at all, effect on targeting the proteins to the thylakoid membranes, e.g. identification in TM preparations, co-purification with PS-II, co-purification of FtsHases (FtsH2 & FtsH5) etc. Yet, if FtsH2 in its mature form consists of one transmembrane segment (as suggested in Rodrigues *et al.,* 2011) then point mutations within TP2 are likely to have rather insignificant effect, unless the cleavage of the N-terminal parts is performed after the main subunits have been clustered together. In this latter case, the effect of point mutations might have an impact on certain characteristics of the enzyme such as stability, performance etc. Regardless however of the timing the disposal of TP2 occurs, if the mature FtsH2 is indeed composed of a single transmembrane segment, then the lumenal domain of 81 amino acids (Bailey *et al.,* 2002), not a loop any more, will be expected to play a significant role in enzyme's activity. Mutational studies within this part of the protein therefore are regarded as essentials if we are to elucidate the role of both domains in the formation and function of the enzyme.

3. Closing thoughts

Since the subject of this research project is in the field of photo-protection, the mechanism that has evolved to counteract the adverse consequences of light, photoinhibition, some thought are shared about our understandings, conventions and the adopted approaches to study these phenomena.

Photosynthesis and photoinhibition, are two radically different processes, that both are driven simultaneously by the same cause, light. The ultimate source of metabolic energy for almost every living cell; the element that regulates the behavior and responses (e.g. migration, leaf senescence, etc) of hundred of thousands of species; the means that connects (vision) thousands of organisms with the world; the dominant force in the life of countless photosynthetic and non-photosynthetic species, appears to be both, beneficial and harmful. The dual effect of light, manifested as photosynthesis and photoinhibition is probably best

reflected in a concept from the mid 16th century, expressed by Paracelsus, a German-Swiss physicist and alchemist, who stated: ‘all things are poison, and nothing is without poison; only the dose permits something not to be poisonous’. A free interpretation of this statement is that the dose, not the substance makes the poison. Similarly, it is not the light that is deleterious to photosynthetic apparatus but the quantity of it.

In the introductory part of this thesis, in an attempt to define and describe photosynthesis as comprehensively as possible, it was stressed that the faculty of photosynthetic apparatus to sense the light and regulate itself accordingly was at least as beautiful and important as the prime function of photosynthesis viz., to synthesize with light. This characteristic ability to ‘read’ the light for self-regulation is not only an intrinsic part of the photosynthetic apparatus, it is above all an inextricable part of it. It is such, because lack of this ‘reading-regulatory‘ capability co-existing with the photosynthetic, would set the existence of the latter rather impossible, at least as it can be deduced from our current understanding of damaging effects of light.

Light appears that does not only produces, but also inhibits the process of photosynthesis itself, and at the same time modulates photosynthesis by regulating the photosynthetic apparatus. Can then photoinhibition be seen as a regulated response rather than a consequence of a mere flaw in the entire system that organisms have not managed to perfect it and have therefore to cope with it? Earlier in this thesis, it was discussed that the concentrated light-induced damage to D1, could be part of a photo-protective mechanism to avoid unregulated damages that would certainly had far greater effect on the organism. Although this idea seems absolutely reasonable, this discourse however is about a greater picture. Where the regulation of photosynthesis ends and where the photoinhibition starts, or where the photoinhibition stands between photoprotection and cell death under certain conditions? How we perceive and define photoinhibition and why? What an approach different from the mainstream convention would bring into our understanding of nature?

In quest to understand better photosynthesis it was established that light does not only drives the photosynthetic machinery to produce but also induces damages to this apparatus that produces. The processes, as it included light was named as photoinhibition. The coined term, photoinhibition, reflects our perception of it, as

something that reduces the productivity and the efficiency of the mechanism. It occurs at all light intensities, but only becomes recordable when it exceeds the capacity of the repair mechanism. The vocabulary dominating the language describing the phenomenon, e.g., damage, reduction, decline in productivity or efficiency, etc, does but reflect our view of the phenomenon as a side effect and merely anything else than that.

How much of an imperfection in the machinery photoinhibition is the result of? How bad is photoinhibition to the organisms? Does photoinhibition (reduced productivity etc) affects to one or another degree the vitality of the cells / organisms? The mere fact that cyanobacteria not only have lived for more than 2.5 billions years, but have thrived for 2.5 billions years, occupying almost every conceivable nook and cranny of this planet is neither a sign of a reduced 'fitness' nor of reduced productivity. Furthermore, the fact that the plants displaying the higher degree to photoinhibition, such as perennial evergreens succeed under the most extreme environmental conditions would hardly be anticipated to be the sign of reduced vitality or productivity. What then Photo Inhibition does to the organism?

Simple rules do underlie nature but they can only be understood when seen through the lenses of nature, not those influenced by our conventional ideas and notions. What does the light-induced damage to the photosynthetic apparatus mean to us and what to the organism itself? It appears that in our perception of the phenomenon, the light-induced damage does indeed lead to reduction of productivity. This definition perfectly reflects our notion of productivity, inasmuch as anything that does in one or another way interfere with the production, anything that does not allow the smooth and unimpaired flow of any process is regarded either as an obstacle or flaw but which nonetheless has to be removed or fixed. The light-generated damages to any protein of the photosynthetic apparatus are seen and treated as such, i.e., imperfections and flaws that impede the productivity. In the world of the living organisms, at least as far the author's knowledge on living organisms allows to anticipate, the pursue for food, either as production in the world of autotrophs or acquisition of it for heterotrophs is desirable only to that extent that will ensure survival and will allow successful transfer of genes to the next generation. Anything more than that characterizes only the world of humans, and maybe some of the creatures

domesticated by them. This idea of what productivity means to people and to the rest of living organisms, concisely expressed through a rephrased notion of Paracelsus (mentioned above), may look like: poison is anything that exceeds the momentary needs of the organism. Given that those needs are defined / sensed only by the organism itself, then anything that leeds to seeming decline of productivity may in fact be a well regulated process. This idea is highly consistent with the suggestion made by Wagner *et al.,* 2004, that what we assumed thus far to be damage may in fact be a mere result of a genetically programmed response.

In a quest to understand nature, man has reduced many of complex phenomena to its basic constituents parts and processes, and then scrutinize them intensely under various laboratory conditions. The aim in this approach is that if we can understand the basic parts then we will understand the whole by reconstructing all the acquired knowledge into one big picture. Although there seems to be no flaw in this strategy that could possibly shake the soundness of this idea, there are nonetheless some questions that merit certain attention. How far we can go in reducing a complex process to its constituents parts? There may be a point, which if we cross with our research, then the process that we think we investigate does not occur at all in the living organism. Then the big picture, that will be composed of such kind of knowledge, how far from the real will be? How far we can go with reducing things and proclaiming afterwards that this is what happens at the base, in a world of a constant flux and interconnectedness. Every process we isolate and study *in vitro,* is never isolated in a living cell. In the world of a living organism, sensing and interacting continuously with its environment, it would not be exaggeration to state that most of the processes that we are dealing with are non-linear, and as such we can only have an approximate knowledge of them. By exposing for instance TM or any other isolated system, to extreme photoinhibitory conditions, we actually strip the organism of its basic ability to react to the challenge. In other words, in these *in vitro* experimentations we do not allow the organism to react by other means other than that we are looking at. No leaf-movement for instance, either result of the wind or re-orientation dictated by the plant; no chloroplast orientation, no positive or negative phototaxis, to name but a few from countless other mechanisms and adaptations that the organism would normally resort to in an attempt to counteract the problem.

Bearing all these ideas and concerns in mind and reconsidering some of our approaches, methods and even the chosen terminology will certainly help us not only to understand better the phenomena we are interested in, but above all to understand them as they occur in nature and not how they would occur if …

Regardless however of how sound the above questions, concerns and suggestions may be, or how useful they may be proved in future practical applications, the perpetual question in Biology of how deep we can understand nature, or if we ever be able to do so, remain enduring and unanswered as ever.

As a final part in this thesis I decided to state the words of a prominent physicist that along with several others laid the foundations of our understanding of matter. The sole reason for doing so, was certainly not due to any pessimistic ideas or beliefs, but simply because comprehension of this reality may help us to understand better the reality itself

"*As physicists have to cope with the uncertainty principle in trying to understand the behavior of an electron, so should biologist face a fundamental limitation when trying to probe an organism too deeply*"
Niels Bohr.

REFERENCES

Adam Z, Adamska I, Nakabayashi K, Ostersetzer O, Haußu¨hl K, Manuell A, Zheng B, Vallon O, Rodermel SR, Shinozaki K, Clarke AK. 2001. Chloroplast and mitochondrial peptidases in Arabidopsis. A proposed nomenclature. Plant Physiol 125:1912– 1918

Adam Z, Clarke AK. 2002. Cutting edge of proteolysis. Trends in Plant Science 7, 451–456.

Adir N., Zer H., Shochat S., Ohad I., 2003. Photoinhibition - a historical perspective. Photosynthesis Research. 76: 343-370.

Akiyama Y., & Ito K. 2000. Roles of multimerization and membrane association in the proteolytic functions of FtsH (HflB). The EMBO J. 19: 3888-3895.

Akiyama Y., Kihara A., Tokuda H., Ito K. 1996. FtsH (HflB) is an ATP-dependent protease selectively acting on SecY and some other membrane proteins. J. Biological Chemistry. 271: 31196-31201.

Akiyama, Y., Kihara, A., and Ito, K. 1996. Subunit a of proton ATPase Fo sector is a substrate of the FtsH protease in Escherichia coli. FEBS Lett **399:** 26–28.

Allen JF, Mullineaux CW, Sanders CE and Melis A 1989. State transitions, photosystem stoichiometry adjustment and non- photochemical quenching in cyanobacterial cells acclimated to light absorbed by Photosystem I or Photosystem II. Photosynth Res 22: 157–166

Allen JF. & Forsberg J. 2001. Molecular recognition in thylakoid structure and function. Trends Plant Scie. 6. 317-326

Allen JF. & Nilson A. 1997. Redox signalling and the structural basis of regulation of photosynthesis by protein phosphorylation. Physiologia Plantarum. 100(4). 863-868

Allen JF. 1992 Protein phosphorylation in regulation of photosynthesis. Biochim Biopsy Acta 1098: 275-335

Allen, M. M. 1984. Cyanobacterial cell inclusions. Annu. Rev. Microbiol. 38:1-25.

Allen, M. M., and A. J. Smith. 1969. Nitrogen chlorosis in blue-green algae. Arch. Mikrobiol. 69:114-120.

Alonso, M., Montoya, G., Cases, R., Rodriguez, R., Picorel, R. 1994. Core antenna complexes, CP43 and CP47, of higher plant photosystem II. Spectral properties, pigment stoichiometry and amino acid composition. *Biochemistry*, **33**, 10494-10500.

Anderson B., and Anderson J.M. 1980. Lateral heterogeneity in the distribution of chlorophyll-protein complexes of the thylakoid membranes of spinach chloroplasts. Biochim. Biophys. Acta. Bionerget. 593:427-440.

Anderson JM. & Melis A. 1983. Localization of different photosystems in separate regions of chloroplast membranes. Proc. Nation. Acad. Scie. USA. 80: 745-749

Anderson JM. 2001. Does functional photosytem II complex have an oxygen channel? FEBS Letters 488. 1-4

Anderson JM., & Anderson B. 1988. The dynamic photosynthetic membrane and regulation of solar energy conversion. Trends Biochim. Sci. 13(9): 351-355.

Anderson JM., & Chow WS. 2002. Structural and functional dynamics of plant photosystem II. Philosoph. Trans. Royal Society. London B. 357(1426): 1421-1430

Anderson SL & McIntosh L. 1991. Light-activated heterotrophic growth of the cyanobacterium Synechocystis sp. strain PCC 6803: a blue-light-requiring process. J. Bactreriology. 173(9). 2761-2767.

Ardelean I, Matthijs HC, Havaux M, Joset F, Jeanjean R. 2002. Unexpected changes in photosystem I function in a cytochrome c6-deficient mutant of the cyanobacterium *Synechocystis* PCC 6803. FEMS Microbiol Lett 213:113–119

Arlt H., Tauer. R., Feldmann H., Neupert W., Langer T. 1996. The YTA10-12 complex, and AAA protease with chaperone like activity in the inner membrane of mitochondria. Cell. 85(6):875-885

Arnon D.I. Photosynthetic electron transport: Emergence of a concept, 1949-1959. Photosynthesis Research. 29(3): 117-131.

Arnoux B., Gaucher JF., Ducruix A., and Reiss-Husson F. 1995. Structure of the photochemical reaction centre of a spheroidene-containing purple bacterium, *Rhodobacter spheroids Y,* at 3Å resolution. Acta Crystalographica D51. 368-379

Arntz B, Trebst A (1986) On the role of the QB protein of PSII in photoinhibition. FEBS Lett 194:43–48.

Aro EM, McCaffery. S., Anderson J.M. 1993. Photoinhibition and D 1 Protein Degradation in Peas Acclimated to Different Growth Irradiances' Plant Physiol. 1993 103, 835-843

Aro EM, Virgin I, Andersson B. 1993. Photoinhibition of Photosystem II. Inactivation, protein damage and turnover. Biochim Biophys Acta. 1143(2):113-34.

Asada K., 1994. Mechanisms fore scavenging reactive molecules generated in chloroplasts under light stress. In Photoinhibition of photosynthesis: From molecular mechanism tot the field. Edited by Baker NR., and Bowler JR. pp 129-142. BIOS Scientific. Oxford

Asada, K. 1999. The water-water cycle in chloroplasts: scavenging of active oxygens and dissipation of excess photons. Annu. Rev. Plant Physiol. Plant Mol. Biol. 50: 601–639.

Ashby MK. & Mullineaux CW. 1999. Cyanobacterial *ycf27* gene products regulate energy transfer from phycobilisomes to photosystems I and II. FEMS Microbiol. Letters. 181(2). 253-260.

Ashby MK. & Mullineaux CW. 1999. The role of ApcD and ApcF in energy transfer from phycobilisomes to PSI and PSII in a cyanobacterium. Photosynth. Research. 61(2). 169-179

Backasch N, Schulz-Friedrich R, Appel J. 2005. Influences on tocopherol biosynthesis in the cyanobacterium *Synechocystis sp*. PCC 6803. J Plant Physiol 162:758–766.

Badger MR. von Caemmerer S. Ruuka S and Nakano H. 2000. Electron flow to oxygen in higher plants and algae: raed and control of direct photoreduction (Mchler reaction) and rubisco oxygenase. Phil Trans. R. Soc. Lond. B. 355: 1433-1446

Baena-González, E., Barbato, R. and Aro, E.-M. 1999. Role of phosophorylation in the repair cycle and oligomeric structure of photosystem II. Planta 208, number 2, pp196-204

Bailey S, Thompson E, Nixon PJ, Horton P, Mullineaux CW, Robinson C, Mann NH. 2002. A critical role for the Var2 FtsH homologue of *Arabidopsis thaliana* in the Photosystem II Repair Cycle in vivo. J. Boil. Chemistry. vol. 277, No 3, Issue January 18. pp 2006-2011

Barbato, R., Race., HL., Friso, G., Barber, J. 1991. Chlorophyll levels in the pigment binding proteins of photosystem II. *FEBS Lett.* **268**, 86-90.

Barber J, Andersson B. 1992. Too much of a good thing: light can be bad for photosynthesis. Trends Biochem Sci. 17(2):61-6.

Barber J. 1992. The photosystems: Structure, Function, and Molecular Biology. Elsevier. Amsterdam.

Barber J., & Archer MD. 2001. P680, the primary electron donor of photosystem II. J. Photochem. Photobiol. 142: 97-106

Barber J., Nield EP., Morris D., Zheleva and Hankamer B. The structure, function and dynamics of photosystem two. Plant Physiol. 100: 817-827

Barber, J. and De Las Rivas , J. 1993. A functional model for the role of cyt-b559 in the protection against donor and acceptor side photoinhibition. Proc. Nat. Acad. Sci. USA. **90**, 10942-10946.

Barber, J., Morris, E.P., Zheleva, D. and Hankamer, B. 1997. The structure, function and dynamics of photosytem two. *Physiol. Plant.* **100**, 817-827

Barker M, Boehm M, Nixon PJ, Nield J. 2008. Structural analysis of an FtsH2/FtsH3 complex isolated from Synechocystis sp. PCC 6803. In: Allen JF, Gantt E, Golbeck JH, Osmond B. eds. Photosynthesis. Energy from the sun. 14th International Congress on Photosynthesis. Springer: The Netherlands, 738–740.

Barker M, de Vries R, Nield J, Komenda J, Nixon PJ. 2006. The Deg pro- teases protect Synechocystis sp PCC 6803 during heat and light stresses but are not essential for removal of damaged D1 protein during the photo- system two repair cycle. J. Biological Chemistry 281: 30347 – 30355.

Baxevanis A., & Ouellette F. 2004. Bioinformatics: a practical guide to the analysis of genes and proteins. 2nd Edition. John Wiley & Sons Inc.

Berger S., Ellersiek U., Steinmüller K. 1991. Cyanobacteria contain a mitochondrial complex I-homologous NADH-dehydrogenase. FEBS letters. 286. 129-132

Berks, B.C., Palmer, T. and Sargent, F. 2003. The Tat protein translocation pathway and its role in microbial physiology. Advances in Microbial Physiology 47, 187-254

Berthold DA., Babcock GT., Yocum CF. 1981. A highly resolved, oxygen-evolving photosystem II preparation from spinach thylakoid membranes. FEBS letters. 134(2): 231-234.

Beyer, A. 1997. Sequence analysis of the AAA protein family. *Protein Sci.* **6,** 2043-2058

Bi E. & Lutkenhaus J. 1991. FtsZ ring structure associated with division in Escherichia coli. Nature 354: 161-164

Biswal B., Smith AJ., Rogers LJ., 1994. Changes in carotenoids but not in D1 protein in response to nitrogen depletion and recovery in a cyanobacterium. FEMS Microbiology Letters. 116 (3), 341-347

Blair, J. E., Shah, P. and Hedges, S. B. 2005. Evolutionary sequence analysis of complete eukaryote genomes. BMC Bioinformatics 6, 53.

Blankenship RE. 1992. Origin and early evolution of photosynthesis. Photos. Research. 33(2): 91-111.

Blankenship RE. 2002. Molecular mechanisms of Photosynthesis. Published by Blackwell Science Ltd.

Blankenship RE., Madigan MT., and Bauer CE. 1995. Anoxygenic Photosynthetic Bacteria. Kluwer Academic Publishers, Dordrecht.

Blankenship RE., Olson JM., Miller M. 1995. Antenna complexes from green photosynthetic bacteria. Advances in photosynthesis. Volume 2. Kluwer Academic Publishers. pp 399-435

Boardman NK, Thorne SW and Anderson JM 1966. Fluorescence properties of particles obtained by digitonin fragmentation of spinach chloroplasts. Proc Natl Acad Sci USA, 56; 586-593.

Bocchi. S., and Malgioglio A., 2010, Azolla-Anabaena as a biofertilizer for rice paddy fields in the Po-Valley, a temperate rice area in northern Italy. International Journal of Agronomy, volume 2010 Article ID 152158, 5 pages

Boehm M., Yu J., Krynicka V., Barker M., Tichy M., Komenda J., Nixon P., Nield J. 2012. Subunit organization of a Synechocystis Hetero-oligomeric thylakoid FtsH complex involved in photosystem II repair. The Plant Cell. 24: 3669-3683

Boekema EJ, Hifney A, Yakushevska AE, Piotrowski M, Keegstra W, Berry S, Michel KP, Pistorius EK, Kruip J (2001) A giant chlorophyll-protein complex induced by iron deficiency in cyanobacteria. Nature 412:745–748

Boekema EJ., Hankamer B., Bald D., Kruip J., Nield J., Boonstra AF., Barber J. and Rögner M. 1995. Supramolecular structure of the photosystem II complex from green plants and cyanobacteria. Proced. Nation. Acad. Scie. USA. 92(1): 175-179.

Boussiba, S., and A. E. Richmond. 1980. C-phycocyanin as a storage protein in the blue-green alga Spirulina platensis. Arch. Microbiol. 125:143-147.

Braatsch S. & Klug Gabriele. 2004. Blue light perception in bacteria. Photosynthesis Research. 79: 45-57

Bricker, T.M., 1990. The structure and function of CPa-1 and Cpa-2 in photosystem II. *Photosynth. Res.* **24**, 1-13.

Bruce D, Brimble S and Bryant DA 1989. State transitions in a phycobilisome-less mutant of the cyanobacterium Synechococcus sp. PCC 7002. Biochim Biophys Acta 974: 66–73

Bryant, D. A, Costas, A. M. G, Maresca, J. A, Chew, A. M. G, Klatt, C. G, Bateson, M. M, Tallon, L. J, Hostetler, J, Nelson, W. C, Heidelberg, J. F, and Ward, D. M. 2007. Candidatus Chloracidobacterium thermophilum: An Aerobic Phototrophic Acidobacterium. Science. 317: 523 - 526

Bryant, D. A. 1986. The cyanobacterial photosynthetic appara- tus: comparisons to those of higher plants and photosynthetic bacteria. Can. Bull. Fish. Aquat. Sci. 214:423-500.

Bullock WO., Fernandez JM., Short JM. 1987. XL-1 Blue: A high efficiency plasmid transforming recA *Escherichia coli* strain with beta-galactosidase selection. Biothechniques. 5(4): 376-379.

Burnap RL, Troyan T, Sherman LA 1993. The highly abundant chlorophyll-protein complex of iron-deficient Synechococcus sp. PCC7942 (CP43') is encoded by the isiA gene. Plant Physiol 103:893–902

Büttner M., Xie DL., Nelson H., Pinther W., Hauska G., Nelson N. Photosynthetic reaction center genes in green sulfur bacteria and in photosystem 1 are related. Proceed. Nation. Acad. Scie. USA. 89. 8135-8139

Byrdin M., Jordan P., Krauss N., Fromme P., Stehlik D., Schlodder E. 2002. Light harvesting in Photosystem I: Modelling based on the 2.5 Å structure of photosystem I form *Synechococcus elongatus.* Biophysical Journal. 83(1): 433-457

Callieri C., Corno G., Caravati E., Galafassi S., Bottinelli M., Bertoni R. 2007. Photosynthetic characteristics and diversity of freshwater *Synechococcus* at two depths during different mixing conditions in a deep oligotrophic lake. J. Limnol. 66(2): 81-89

Carpenter, SD., Ohad, I., Vermaas, WFI., 1993. Analysis of chimeric spinach/ cyanobacterial CP43 mutants of *Synechocystis* sp. PCC6803: the chlorophyll-protein CP43 affects the water-splitting system of Photosystem II. *Biochim. Biophys. Acta.* **1144**, 204-212.

Castenholtz RW. 1988. Culturing methods for cyanobacteria. Methods in Enzymology. 167: 68-93. Ed. Packer L and Glazer AN., Academic Press, San Diego.

Castenholz, R.W. and Waterbury, J.B. 1989. Preface. In: Staley, J.T., Bryant, M.P., Pfenning, N., Holt, JG. (eds) Bergey's manual of systematic bacteriology, Vol 3. Williams and Wilkins, Baltimore, pp 1710-1727.

Catenholz, R.W. and Waterbury, J.B. 1989. Taxa of the Cyanobacteria. In: Staley, J.T., Bryant, M.P., Pfenning, N., Holt, JG (eds)Bergey's manual of systematic bacteriology. Vol 3. Williams and Wilkins, Baltimore, pp 1727-1728

Cavalier-Smith, T. 1999. Principles of protein and lipid targeting in secondary symbiogenesis: Euglenoid, dinoflagellate, and sporozoan plastid origins and the eukaryote family tree. Journal of Eukaryotic Microbiology 46, 347–366.

Chen M., Choi YD., Voytas DF., Rodermel S. 2000. Mutations in the Arabidopsis VAR2 locus cause leaf variegation due to loss of a chloroplast FtsH. The Plant J. 22(4): 303-313

Cheng LJ., Ma JS., Chiang LC., 1990. The complexes formed by biladiene *a, b* compounds with zinc ions and their application in determination of the chromophore composition of *a*-and -*b* subunits of r-phycoerethrin. Photochem. Photobiol. 52(6). 1071-1076.

Chitinis, PR. 1996. Photosystem I. *Plant Physiology.* **111**, 661-669.

Chitnis PR & Thornber JP 1988. The major light-harvesting couplex of Photosystem II: aspects of its molecular and cell biology. Photosyn. Research. 16. 41-63

Chitnis, P.R., Xu, Q., Chitnis, V. and Nechustai, R. 1995. Function and organization of Photosystem-I polypeptiedes. Photosynth. Res. 44, 23^40

Chitnis, PR.2001. Photosystem I. Function and physiology. *Ann. Rev. Plant Physiol. Plant Mol. Biol.***52**, 593-626.

Chow WS., Hope AB., Anderson JM., 1990. A reassessment of the use of herbicide binding to measure photosystem II reaction centers in plant thylakoids. Photosynthesis Research 24(1), 109-113.

Chow WS., Osmond CB., Huang LK., 1989. Photosystem II function and herbicide binding sites during photoinhibition of spinach chloroplasts in-vivo and in-vitro. Photosynthesis Research. 21(1). 17-26.

Chua NH., and Gillham NW. 1977. The sites of synthesis of the principal thyalakoid membrane polypeptides in *Chlamydomonas reinhardtii.* J. Cel. Biol. 74(2): 441-452.

Ciferri O. 1983. Spirulina,the Edible Microorganism. Microbiological Reviews. 47: 551-578

Cogdell R., & Frank HA. 1987. How carotenoids function in photosynthetic bacteria. Biochim. Biophys. Acta. Review Bioenerg. 895(2): 63-79

Collier J & Grossman A 1992. Chlorosis Induced by Nutrient Deprivation in Synechococcus sp. Strain PCC 7942: Not All Bleaching Is the Same. J. Bacteriol. Vol 174, 4718-4726

Collins AM, Liberton M, Jones HDT, Garcia OF, Pakrasi HB, and Timlin JA. 2012. Photosynthetic pigment localization and thylakoid membrane morphology are altered in *Synechocystis* 6803 phycobilisome mutants. Plant Physiology Preview.

Confalonieri F., Duguet M., 1995. A 200-amino acid ATPase module in search of a basic function. BioEssays. 17(7):639-650

de Marsac NT & Cohen-Bazire G. 1977. Molecular composition of cyanobacterial phycobilisomes. Proc. Nation. Acad. Sci. USA. 74(4). 1635-1639

De Rosa M, Gambacorta A, Gliozzi A. 1986. "Structure, biosynthesis, and physicochemical properties of archaebacterial lipids". Microbiol. Rev. 50 (1): 70–80.

Debus RJ. 1992. The manganese and calcium ions of photosynthetic oxygen evolution. Biochim. Biophys. Acta. 1102(3): 269-352

Dekker JP., & Boekema EJ. 2005. Supramolecular organization of thylakoid membrane proteins in green plants. Biochem. Biophys. Acta. 1706: 12-39

Dekker, J.P., Bowlby, N.R., Yocum, CF., Boekema, EJ. 1990. Characterization by electron microscopy of isolated particles an two-dimensional crystals of the CP47-D1-D2-cytochrome b-559 complex of Photosystem II. *Biochemistry*, **29**, 3220-3225

Demmig-Adams B & Adams WW., 1992. Photoprotection and other responses of plants to high light stress. Ann. Rev. Plant Physiol. Plant mol. Biol. 43: 599-626.

Demmig-Adams, B. and Adams, W.W. III. 1996. The role of the xanthophyll cycle carotenoids in protection of photosynthesis. Trends. Plant Sci. 1: 21–26.

Deuerling, E., Mogk, A., Richter, C., Purucker, M., and Schu- mann, W. 1997. The ftsH gene of Bacillus subtilis is involved in major cellular processes such as sporulation, stress adaptation and secretion. Mol Microbiol 23: 921– 933.

Deusch O.,Landan G., Roettger M., Gruenheit N., Kowallik KV,, Allen JF., Martin W., Dagan T., 2008. Genes of Cyanobacterial Origin in Plant Nuclear Genomes Point to a Heterocyst-Forming Plastid Ancestor. Mol. Biol. Evol. 25 (4): 748-761.

Devaki B, Grossman AR, Steunou AS, Khuri N, Cohan FM, Hamamura N, Melendrez MC, Bateson MM, Ward DM, Heidelberg JF. 2007. Population level functional diversity in a microbial community revealed by comparative genomic and metagenomic analyses. ISME J. 1(8):703-713. Epub 2007 Oct 25

Diner BA., Nixon PJ., Farchaus JW., 1991. Site-directed mutagenesis of photosynthetic reaction centers. Current Opinion in Struc Biol. vol 1(4), 546-554

Donaher N, Tanifuji G, Onodera NT, Malfatti SA, Chain PS, Hara Y, Archibald JM. 2009. The complete plastid genome sequence of the secondarily nonphotosynthetic alga Cryptomonas paramecium: reduction, compaction, and accelerated evolutionary rate.Genome Biol. Evol. 13: 439-448.

Douglas S, Zauner S, Fraunholz M, Beaton M, Penny S, Deng LT, Wu XN, Reith M, Cavalier-Smith T, Maier UG. 2002. The highly reduced genome of an enslaved algal nucleus. Nature 410: 1091-1096

Ducret A., Sidler W., Frank G., Zuber H., 1994. The complete amino acid sequence of R-phycocyanin-I α and β subunits from the red alga *Porphyridium cruentum.* Eur. J. Biochem. 221. 563-580.

Durrant JR., Klug DR., Kwa SL., van Grondelle R., Porter G., and Dekker JP. A multimer model for P680, the primary electron donor of photosystem II. Proc. Nation. Acad. Sci. USA. 92: 4798-4802

Edelman M, Mattoo AK. 2008. D1-protein dynamics in photosystem II: the lingering enigma. Photosynthesis Research 98: 609–620.

Edwards MR. & Gantt. E. 1971. Phycobilisomes of thermophilic blue-green alga *Synechococcus lividus.* J. Cell Biol. 50. 896-900

Elhai J., & Wolk PC. 1988. A versatile class of positive-selection vectors based on the non viability of palindrome-containing plasmids that allows cloning into long polylinkers. Gene. 68(1): 119-138

Emlyn-Jones D., Ashby MK., Mullineaux CW., 1999. A gene required for the regulation of photosynthetic light harvesting in the cyanobacterium *Synechocystis* 6803. Mol. Microbial. 33: 1050-1058.

Endo T., Mil H., Shikanai T., Asada K. 1997. Donation of electrons to plastoquinone by NAD(P)H dehydrogenase and by ferrodoxin-quinone reductase in spinach chloroplasts. Plant Cell Physiol. 38(11): 1272-1277.

Endo T., Shikanai T., Takabayashi A., Asada K., Sato F. 1999. The role of chloroplastic NAD(P)H dehydrogenase in photoprotection. FEBS Letters. 457(1): 5-8

Erickson JM., Rahire M., Bennoun P., Delepelaire P., Diner B., and Rochaix JD. 1984. Herbicide resistance in *Chlamydomonas reinhardtii* results from a mutation in the chloroplast gene for the 32-kilodalton protein of photosystem II. Proc. Nat. Acad. Science USA. 81. 3617-3621

Erickson JM., Rahire M., Rochaix JD., Mets L. 1985. Herbicide resistance and cross-resistance: changes at three distinct sites in the herbicide-binding protein. Science. 228. 204-207

Erickson, H.P., 1998. Atomic structures of tubulin and FtsZ .Trends in Cell Biology. 8: 133-137.

Falk S, Samson G, Bruce D, Huner NP 1995. Functional analysis of the iron-stress induced CP43' polypeptide of PS II in the cyanobacterium *Synechococcus sp*. PCC 7942. Photosynth Res 45:51–60

Faller P., Debus RJ., Brettel K., Sugiura M., Rutherford AW., Boussac A. 2001b. Proc. Nation. Acad. Scie. USA.98: 14368-14373,

Faller P., Pascal A., William A. 2001. *β*-carotene redox reactions in photosystem II: electron transfer pathway. Biochemistry. 40(21): 6431-6440.

Fawley MW., Grossman AR. 1986. Polypeptides of a light-harvesting complex of the diatom *Phaeodactylum tricornutum* are synthesised in the cytoplasm of the cell as precursors. Plant Physiology. 81(1). 149-155.

Ferris M.J., and Palenik B. 1998 Niche adaptation in ocean cyanobacteria *Nature* **396**, 226-228

Field D, Garrity G, Gray T, Morrison N, Selengut J, Sterk P, Tatusova T, Thomson N, Allen MJ, Angiuoli SV, Ashburner M, Axelrod N, Baldauf S, Ballard S, Boore J, Cochrane G, Cole J, Dawyndt P, De Vos P, DePamphilis C, Edwards R, Faruque N, Feldman R, Gilbert J, Gilna P, Glöckner FO, Goldstein P, Guralnick R, Haft D, Hancock D, Hermjakob H, Hertz-Fowler C, Hugenholtz P, Joint I, Kagan L, Kane M, Kennedy J, Kowalchuk G, Kottmann R, Kolker E, Kravitz S, Kyrpides N, Leebens-Mack J, Lewis SE, Li K, Lister AL, Lord P, Maltsev N, Markowitz V, Martiny J, Methe B, Mizrachi I, Moxon R, Nelson K, Parkhill J, Proctor L, White O, Sansone SA, Spiers A, Stevens R, Swift P, Taylor C, Tateno Y, Tett A, Turner S, Ussery D, Vaughan B, Ward N, Whetzel T, San Gil I, Wilson G, Wipat A. 2008. The minimum information about a genome (MIGS) specification. Nat. Biotechnol. 26(5): 541-547

Flors C., Fryer MJ., Waring J., Reeder B., Bechtold U., Mullineaux PM., Nonell S., Wilson MT., Baker NR.2006. Imaging the production of singlet oxygen in vivo using a new fluorescent sensor, Singlet Oxygen Sensor GreenR. J Exp Bot 57:1725–1734.

Fork DC., & Herbert SK. 1993. Electron transport and phosphorylation by Photosystem I *in vivo* in plants and cyanobacteria. Photosynthesis Research. 36(3): 149-168.

Foster JS, Singh AK, Rothschild LJ, Sherman LA. 2006. Growth- phase dependent differential gene expression in *Synechocystis sp.* strain PCC 6803 and regulation by a group two sigma factor. Dig Arch Microbiol DOI 10. 1007/s0020300601936

Foyer CH, Trebst A, Noctor G. 2006. Signaling and integration of defense functions of tocopherol, ascorbate and glutathione. In: Demmig-Adams B, Adams WWIII, Mattoo AK (eds) Photoprotection, photoinhibition, gene regulation and environment. Springer, The Netherlands, pp 240–268

Frank HA., and Cogdell RJ. 1996. Carotenoids in photosynthesis. Photochem. Photobiol. 63(3): 257-264

Friedman AL. & Alberte RS. 1986. Biogenesis and light regulation of the major light harvesting chlorophyll-protein of diatoms. Plant Physiol. 80(1). 43-51

Fromme P., Jordan P., Krauss N. 2001. Structure of photosystem I. Biochim. Biphys. Acta - Bioenerg. 1507: 5-31.

Fujita Y, Murakami A, Aizawa K, Ohki K. 1994. Short-term and Long-term Adaptation of the Photosynthetic Apparatus: Homeostatic Properties of Thylakoids. In DA Bryant, ed, The Molecular Biology of Cyanobacteria, Vol 1. Springer Netherlands, pp 677-692

Fujita Y, Ohki K and Murakami A. 1985. Chromatic regulation of photosystem composition in the photosynthetic system of red and blue-green algae. Plant Cell Physiol 26: 1541–1548

Fujita Y. 1996. Flexibility of energy conversion processes in thylakoids of cyanobacteria. J. Scientific & Industrial Res. 55: 618-629

Funk C, Vermaas W. 1999. A cyanobacterial gene family coding for single- helix proteins resembling part of the light-harvesting proteins from higher plants. Biochemistry 38: 9397–9404.

Funk, C. 2000. Functional analysis of the PsbX protein by deletion of the corresponding gene in *Synechocystis* sp PCC 6803. *Plant Mol. Biol.* **44**, 815-827.

Gantt E. & Conti SF. 1969. Ultrastructure of Blue-Green Algae. J. Bacteriol. 97(3). 1486-1493

Gantt E. 1975. Phycobilisomes: Light-Harvesting Pigment Complexes. BioScience. 25 (12). 781-788.

Gantt E., Edwards MR., Provasoli L. 1971. Chloroplast structure of the cryptophyceae. Evidence for phycobiliproteins within intrathylakoid spaces. J. Cell Biol. 48(2). 280-290.

Gantt. E. 1981. Phycobilisomes. Ann.Review. Plant Physiol. 32. 327-347.

Gao H., Xu X. 2009. Depletion of Vipp1 in Synechocystis sp. PCC 6803 affects photosynthetic activity before the loss of thylakoid membranes. FEMS Microbiology Lett. 292: 63-70

Gasanov R, Abilov ZK, Gazanchyan RM, Kurbonova UM, Khanna R and Govindgee 1979. Excitation energy transfer in Photosystem I and II from grana and in Photosystem I from stroma lamellae, and identification of emission bands with pigment protein complexes. eZit Pflanzenphysiol 95: 148-169

Geider RJ, Roche J., Greene RM., Olaizola M. 1993. Response of the photosynthetic apparatus of Phaeodactylm tricornutum (Bacillariophyceae) to nitrate, phosphate or iron starvation. J. Phycology, 29(6) 755-766.

Ghanotakis, D.F., de Paula, J.C., Demetriou, D.M., Bowly, NR., Peterson J., Babcock, GT. and Yocum, C.F.1989. Isolation and characterization fo the 47 kDa protein and the D1-D2 cytochrom b-559 complex. *Biochim. Biophys. Acta.* **974**, 44-53

Ghosh & Govindjee 1966. Transfer of the excitation energy in Anacystis nidulans grown to obtain different pigment ratios. Biopsy. J. 6, 611-619

Gilmore AM, Itoh SS and Govindjee 2000. Global spectral-kinetic analysis of room temperature chlorophyll a fluorescence from light harvesting antenna mutants of barley. Phil Trans Roy Soc London B335, 1-14.

Glaser F., Pupko T., Paz I., Bell RE., Bechor-Shental D., Martz E., & Ben-Tal N. 2003. Con-surf: identification of functional regions in proteins by surface-mapping of phylogenetic information. Bioinformatics. 19: 163-164.

Glazer AN. 1985. Light harvesting by phycobilisomes. Ann. Rev. Biophys. Biophys. Chemistry. 14. 47-77.

Glazer AN., Yeh SW., Webb SP., Clark JH. 1985. Disk-to-disk transfer as the rate-limiting step for energy flow in phycobilisomes. Science. 227. 419-423.

Glazer AN., Chan C., Williams RC., Yeh SW., Clark JH. 1985. Kinetics of energy flow in the phycobilisome Core. Science. 230. 1051-1053.

Glazer, A. 1994. Phycobiliproteins – a family of valuable, widely used fluorophores. *J. Applied Phycol.* **6**, 105-112.

Golden, S., Nalty MS, and Cho DSC. 1989. Genetic relationship of two highly studied Synechococcus strains designated Anacystis nidulans. J. Bacteriol. 171:24-29.

Golyshina OV, Pivovarova TA, Karavaiko GI, T F Kondratéva, Moore ER., Abraham WR., Lünsdorf H, Timmis KN., Yakima MM., and Golyshin PN. 2000. "Ferroplasma acidiphilum gen. nov., sp. nov., an acidophilic, autotrophic, ferrous-iron-oxidizing, cell-

wall-lacking, mesophilic member of the Ferroplasmaceae fam. nov., comprising a distinct lineage of the Archaea". Int. J. Syst. Evol. Microbiol. 50 (3): 997–1006.

Gottesman, S., Wickner, S. and Maurizi, M.R. 1997. Protein quality control: triage by chaperones and proteases. Genes Dev. 11, 815±823.

Gould SB, Waller RF, McFadden GI. 2008. Plastid evolution. Annu. Rev. Plant. Biol. 59: 491-517

Govindjee and Yang L 1966 Structure of the red fluorescence band in chloroplasts. J. Gen Physiol 49: 763-780.

Govindjee, Ames J and Fork DC 1986. Light Emission by Plants and Bacteria. Academic Press, Orlando.(Early volume on Chlorophyll fluorescence)

Granger LL, O'Hara EB., Wang R.-F., Meffen, FV., Armstrong, K., Yancy, S.D., Babitzke, P., and Kushner, S.R. 1998. The *Escherichia coli mrsC* gene is required for cell growth and mRNA decay. J Bacteriol 180: 1920–1928.

Green BR. 2003. The evolution of light harvesting Antennas. in: Advances in Photosynthesis and Respiration Volume 13. Light-Harvesting Antennas in Photosynthesis. Kluwer Academic publishers 129-168

Green BR., Pichersky E., Kloppstech K. 1991. Chlorophyll *a/b*-binding proteins: an extended family. Trends Biochem. Scie. 16. 181-186

Grigorieva G., & Shestakov S. 1982. Transformation in the cyanobacterium *Synechocystis sp.* 6803. FEMS Letters. 13(4): 367-370.

Grossman A., Manodori A., Snyder D., 1990. Light-harvesting proteins of diatoms: their relationship to chlorophyll *a/b* binding proteins of higher plants and their mode of transport into plastids. Molecul. General Genetics. 224(1). 91-100

Grossman AR, Van Waasbergen L, Schwarz S. 2000. Impact of stress conditions on the photosynthetic machinery. J Phycology. vol.36, issue September, p 26-27

Grossman AR. 1990. Chromatic adaptation and the events involved in phyobilisomes biosynthesis. Plant Cell Environment. 13(7). 651-666

Grossman AR. 1993. The phycobilisome, a light-harvesting complex responsive to environmental conditions. Microbiol Rev 57:725–749

Grossman AR. Devaki B., Apt KE., Kehoe DM. 1995. Light-harvesting complexes in oxygenic photosynthesis: Diversity, Control, and Evolution. Ann. Rev. Genetics. 29. 231-288

Gupta R. S. 1998. "Protein phylogenies and signature sequences: A reappraisal of evolutionary relationships among archaebacteria, eubacteria, and eukaryotes". Microbiol. Mol. Biol. Rev 62 (4): 1435–1491.

Gupta RS. 2000. The phylogeny of proteobacteria: relationships to other eubacterial phyla and eukaryotes. FEMS Microbiol Rev. 24(4):367-402

Gupta RS. 2005. Molecular sequences and the early history of life (Chapter 8). In 'Microbial phylogeny and Evolution: concepts and controversies. New York. Oxford University Press.

Gupta RS., Chander P., George S. 2012. Erratum to: Phylogenetic framework and molecular signatures for the class Chloroflexi and its different clades; proposal for division of the class Chloroflexi class. nov. into the suborder Chloroflexineae subord. nov., consisting of the emended family Oscillochloridaceae and the family Chloroflexaceae fam. nov., and the suborder Roseiflexineae subord. nov., containing the family Roseiflexaceae fam. nov. Antonie van Leeuwenhoek. 113(1): 261

Guskov A., Kern J, Gabdulkhakov A., Broser M., Zouni A., Saenger W. 2009. Cyanobacterial photosystem II at 2.9-Å resolution and the role of quinones, lipids, channels and chloride. *Nature Structural & Molecular Biology* **16**, 334 - 342

Hagemann M., Schoor A., Jeanjean R., Zuther E., and Joset F. The stpA gene from *Synechocystis sp.* strain PCC 6803 encodes the glucosylglycerol-phosphate phosphatase involved in cyanobacterial osmotic response to salt shock. J. Bacteriology. 179(5): 1727-1733.

Hankamer B., Barber J., and Boekema EJ. 1997. Structure and membrane organization of photosystem II in green plants. Ann. Rev. Plant Physiol. Plant Molec. Biol. 48: 641-671.

Hankamer, B., Morris, E.P. and Barber, J. 1999. Revealing the structure of the oxygen-evolving core dimer of photosystem II by cryoelectron crystallography. *Nature Struc. Biol*. **6**, 560-564.

Hanley, J., Deligiannakis Y., Pascal A., Faller P. and Rutherford AW. 1999. Carotenoid oxidation in photosystem II. Biochemistry, 38. 8189-8195.

Harper, J. T. & Keeling, P. J. 2003. Nucleus-encoded, plastid-targeted glyceraldehyde-3-phosphate dehydrogenase (GAPDH) indicates a single origin for chromalveolate plastids. Molecular Biology and Evolution 20, 1730–1735.

Haumann, M. and Junge, W. 1999. Photosynthetic water oxidation: a simplex-scheme to its partial reactions. Biochim. Biophys. Acta 1411, 86-91.

Haupts, U., Tittor, J. and Osterhelt, D. 1999. Closing in on bacteriorodopsin: Progress in understanding the molecule. Ann. Rev. Biophys. Biomol. Struct. **28**, 367-399

Hauschild, C.A., H.J.G McMurter & F.R. Pick. 1991. Effect of spectral quality on growth and pigmentation of pico- cyanobacteria. J. Phycol., 27: 698-702.

Havaux M, Eymery F, Porfirova S, Rey P, Dormann P. 2005. Vitamin E protects against photoinhibition and photooxidative stress in Arabidopsis thaliana. Plant Cell 17:3451–3469.

Havaux M, Guedeney G, Hagemann M, Yeremenko N, Matthijs HC, Jeanjean R. 2005. The chlorophyll-binding protein IsiA is inducible by high light and protects the cyanobacterium *Synechocystis* PCC6803 from photooxidative stress. FEBS Lett 579:2289–2293

Heber U., & Walker D. 1992. Concerning a dual function of coupled cyclic electron transport in leaves. Plant Physiol. 100(4): 1621-1626.

Herbert SK., Martin RE., Fork DC. Light adaptation of cyclic electron transport through photosystem I in the cyanobacterium *Synechococcus sp.* PCC7942. Photosynthesis Research. 46: 277-285.

Hippler, M., Rimbault, B., and Takahashi, Y. (2002). Photosynthetic complex assembly in Chlamydomonas reinhardtii. Protist 153, 197–220.

Hiyama T., & Ke B. 1972. Difference spectra and extinction coefficients of P700. Biochim. Biphys. Acta - Bioenrget. 267(1):160-171

Hoganson CW., and Babcock GT. 1997. A Metalloradical Mechanism for the Generation of Oxygen from Water in Photosynthesis. Science 277: 1953-1956

Holzwarth AR 1991. Excited state kinetics in chlorophyll systems and its relationships to the functional organization of the photosystems. In: Scheer H (ed) Chlorophylls, pp 1125-1151.

Honda D., Yokota A., Suglyama J. 1999. Detection of seven major evolutionary lineages in cyanobacteria based on the 16rRNA gene sequence analysis with new sequences of five marine Synechococcus strains. J. Molec. Evol. 48:723-739

Horton P., & Ruban AV. 2005. Molecular design of the photosystem II light-harvesting antenna: photosynthesis and photoprotection. J. Exp. Bot. 56 (411): 365-373

Horton P., Ruban AV., Wentworth M. 2000. Allosteric regulation of the light-harvesting system of photosystem II. Phil. Trans. R. Soc. Lond. B. 355. 1361-1370

Horton, P., Ruban, A.V. and Walters, R.G. 1996. Regulation of light harvesting in green plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 47: 655–684.

Hoshida, H., Sugiyama, R., Nakano, Y., Shiina, T. and Toyoshima, Y. 1997. Electron paramagnetic resonance and mutational analyses revealed the involvement of photosystem II – L subunit in the oxidation step of Tyr-Z by $P680^{+}$ to form the $TyrZ^{+}$ P680 $Pheo^{-}$ state in photosystem II. *Biochemistry*. **36**, 12053-12061.

Hugueney P, Bouvier F, Badillo A, D'Harlingue A, Kuntz M, Camara B. 1995. Identification of a plastid protein involved in vesicle fusion and/ or membrane protein translocation. Proc Natl Acad Sci USA 92: 5630–5634

Huner NPA, Ögust G and Sahran F 1998. Energy balance and acclimation to light and cold. Trends in Plant Science 3: 224-230

Huson. D.H. and Bryant, D. 2006. Application of Phylogenetic Networks in Evolutionary Studies, *Mol. Biol. Evol*., **23**(2):254-267.

Ikeuchi M, Eggers B, Shen G, Webber A, Yu J. 1991. Cloning of the psbK gene from Synechocystis sp. PCC 6803 and characterization of photosystem II in mutants lacking PSII-K. J. Biol. Chem. 266:11111-15

Imanian B., Pombert JF., Keeling PJ. 2010. The complete plastid genomes of the two 'dinotoms' Durinska baltica and Kryptoperidinium fallaceum. PLoS One. 19(5): e10711

Inoue K, Sakura H & Hiyama T. 1986. Photoinactivation sites of photosystem I in Isolated chloroplasts. Plant & Cell Physiology. 27(6): 961-968

Jegerscho ̈ld C, Virgin I, Styring S. 1990. Light-dependent degrada- tion of the D1 protein in photosystemII is accelerated after inhibition of the water splitting reaction. Biochemistry 29:6179– 6186.

Johnson, P. W., Hargraves, P. E. & Sieburth, J. M. 1988. Ultrastructure and ecology of Calycomonas ovalis Wulff, 1919, (Chrysophyceae) and its redescription as a testate Rhizopod, Paulinella ovalis N. Comb. (Filosea: Euglyphina). Journal of Protozoology 35, 618–626

Jordan P., Fromme P., Witt HT., Klukas O., Saenger W., Kraus N., Three dimensional structure of cyanobacterial photosystem I at 2.5 Å resolution. Nature. 411: 909-916.

Joshua, S., and Mullineaux, C.W. (2005). The rpaC gene product regulates phycobilisome-photosystem II interaction in cyanobacteria. Biochim. Biophys. Acta 1709, 58–68.

Jursinic P., & Stemler A., 1983. Changes in 14C Atrazine binding associated with the oxidation-reduction state of the secondary quinone acceptor of photosystem II. Plant Physiol. 73, 703-708

Kamiya, N. and Shen, J-R. 2003. Crystal structure of oxygen evolving photosystem II from *Thermosyvnechococcus vulcanus* at 3,7 Å resolution. *Proc. Natl. Acad. Sci. USA*. 100, 98-103.

Kaneko T., Sato S., Kotani H., Tanaka A., Asamizu E., Nakamura Y., Miyajima N., Hirosawa M, Sugiura M., Sasamoto S., Kimura T., Hosouchi T., Matsuno A., Muraki A., Nakazaki N., Naruo K, Okumura S, Shimpo S., Takeuchi C., Wada T., Watanabe A., Yamada M., Yasuda M., Tabata S. 1996. Sequence Analysis of the Genome of the Unicellular Cyanobacterium Synechocystis sp. Strain PCC6803. II. Sequence Determination of the Entire Genome and Assignment of Potential Protein-coding Regions. DNA Research 3: 109-126

Karata K, Inagawa T, Wilkinson AJ, Tatsuta T, Ogura T. 1999 Dissecting the role of a conserved motif (the second region of homologuey) in the AAA family of ATPases. Site-directed mutagenesis of the ATP-dependent peptidase FtsH. J Biol Chem 274:26225–26232

Kasahara M, Kagawa T, Oikawa K, Suetsugu N, Miyao M &Wada M. 2002. Chloroplast avoidance movement reduces photodamage in plants. Nature 420. 829-832.

Kato Y, Miura E, Ido K, Ifuku K, Sakamoto W. 2009. The variegated mutants lacking chloroplastic FtsHs are defective in D1 degradation and accumulate reactive oxygen species. Plant Physiology 151: 1790 – 1801.

Keren N, Ohkawa H, Welsh EA, Liberton M, Pakrasi HB. 2005. Psb29, a conserved 22-kD protein, functions in the biogenesis of photosystem II complexes in *Synechocystis* and Arabidopsis. The Plant Cell 17: 2768 – 2781.

Kerfeld CA. 1997. Structural comparison of cytochrome C2 and cytochrom C6. Photosynthesis Research. 54(2):81-98.

Kihara A., Akiyama A., and Ito, K. 1998. Differential pathways for protein degradation by the FtsH/HflKC membrane-embedded protease complex: an implication from the interference by a mutant form of a new substrate protein, YccA. J. Mol. Biol. 279: 175-188

Kihara A., Akiyama Y., & Ito K. 1996. A protease complex in the Escherichia coli plasma membrane: HflKC (HflA) froms a complex with FtsH (HflB), regulating its proteolytic activity against SecY. EMBO J. 15: 6122-6133

Kim E, Lane CE, Curtis BA, Kozera C, Bowman S, Archibald JM. 2008. Complete sequence and analysis of the mitochondrial genome of Hemiselmis andersenii CCMP644 (Cryptophyceae). BMC Genomics. 12;9:215.

Kirilovsky D. 2007. Photoprotection in cyanobacteria: the orange carotenoid protein (OCP)- related non-photochemical-quenching mechanism. Photosynth. Res. 93: 7-16.

Kitamura K, Ozawa S, Shiina T, Toyoshima Y. 1994. L protein, encoded by psbL, restores normal functioning of the primary quinone acceptor, QA, in isolated D1/D2/ CP47/Cytb-559/I photosystem II reaction center core complex. FEBS Lett. 354:113-116

Klimov VV., Allakhverdiev SI., Demeter Sh., Krasnovski AA. 1979. Pheophytin reduction in photosystem II of [pea] chloroplasts in relation to oxidation-reduction potential of medium. Doklady biophysics. 247:190-192.

Knaff DB. 1996. in *Oxygenic Photosynthesis: The Light Reactions. eds. Ort, DR. & Yocum, CF. Kluwer Academic Publishers,Dordrecht. Vol.4: 333-361*

Koga Y, & Morii H. 2007. "Biosynthesis of ether-type polar lipids in archaea and evolutionary considerations". Microbiol. Mol. Biol. Rev. 71 (1): 97–120.

Kok B., Forbush B., and McGloin M. 1970. Cooperation of charges in photosynthetic O2 evolution-I. A linear four step mechanism. Photochem. Photobiol. 11(6): 4557-475.

Koksharova OA., Klint J., Rasmussen U. 2006. The first protein map of Synechococcus sp. strain PCC7942. Microbiology. 75(6): 664-672.

Komenda J, Barber J. 1995. Comparison of psbO and psbH deletion mutants of *Synechocystis* PCC 6803 indicates that degradation of D1 protein is regulated by the QB site and dependent on protein synthesis. Biochemistry 34, 9625–9631.

Komenda J., Barker M., Kuvikova S., deVries R., Mullineaux CW., Tichy M., Nixon PJ. 2006. The FtsH protease slr0228 is important for quality control of photosystem II in the thylakoid membrane of *Synechocystis* sp PCC 6803. Journal of Biological Chemistry 281: 1145–1151

Komenda J., Tichy M., Prášil O., Knoppova J., Kuvikova S., deVries R., Nixon PJ. 2007. The exposed N-terminal tail of the D1 subunit is required for rapid D1 degradation during photosystem II repair in *Synechocystis* sp PCC 6803. The Plant Cell 19: 2839–2854.

Komenda, J. and Barber, J. 1995. Comparison of psbO and psbH Deletion mutants of *Synechocystis* PCC 6803 indicates that the degradation of D1 protein is regulated by the Q_B site and Dependent of protein sysnthesis. *Biochemistry*, **34**, 9625-9631

Kozaki, A. and Takeba, G. 1996. Photorespiration protects C3 plants from photooxidation. Nature 384: 557–560.

Krause GH and Weis E 1991. Chlorophyll fluorescence and photosynthesis: The basics. Ann Rev Plant Physiol Mol Biology. 42: 313-349.

Kreiger-Liszkay A, Trebst A. 2006. Tocopherol is the scavenger of singlet oxygen produced by the triplet states of chlorophyll in the PSII reaction center. J Exp Bot 57:1677–1684.

Kreiger-Liszkay A, Trebst A. 2006. Tocopherol is the scavenger of singlet oxygen produced by the triplet states of chlorophyll in the PSII reaction center. J Exp Bot 57:1677–1684.

Kuhlbrandt, W., Wang, DN., Fujiyoshi, Y. 1994. Atomic model of plant light-harvesting complex by electron crystallography. *Nature*. **367**, 614-621

Kyle DJ, Ohad I and Arntzen CJ. 1984. Membrane protein damage and repair: selective loss of quinone-protein function in chloro- plast membranes. Proc Nation. Acad Sci USA 181: 4070–4074

Lambers H., Chapin III FS., and Pons TL. 2008. in Plant Physiological Ecology. 2nd edition. Springer Science Business Media. p 13-15.

Lane CE, van den Heuvel K, Kozera C, Curtis BA, Parsons BJ, Bowman S, Archibald JM.. 2007. Nucleomorph genome of Hemiselmis andersenii reveals complete intron loss and compaction as a driver of protein structure and function. Proc Natl Acad Sci U S A. 1;104(50):19908-13.

Langer T., 2000 AAA proteases: cellular machines for degrading membrane proteins. Trends n Biochem. Scien. Volume 25, Issue 5, 247-251

Lardans A., Gillham W., Boynton JE. 1997. Site-directed mutations at residue 251 of the photosystem II D1 protein of Chlamydomonas that result in a nonphotosynthetic phenotype and impair D1 synthesis and accumulation. J. Biol Chem. 272. 210-216.

Larkum AWD. & Barrett J., 1983. Light-harvesting processes in algae. Advances Botanical Research. 10. 1-219.

Lau, R H., M. M. MacKenzie, and W. F. Doolittle. 1977. Phycocyanin synthesis and degradation in the blue-green bac- teriumAnacystis nidulans. J. Bacteriol. 132:771-778.

Leander, B. S., Triemer, R. E. and Farmer, M. A. 2001. Character evolution in heterotrophic euglenids. Eur. J. Protistol. 37:337-356.

Leander, B.S. 2004. Did trypanosomatid parasites have photosynthetic ancestors? Trends Microbial. 12:251-258.

Li H, Singh AK, McIntyre LM, Sherman LA. 2004. Differential gene expression in response to hydrogen peroxide and the putative PerR regulon of *Synechocystis sp*. strain PCC 6803. J Bacteriol 186:3331–3345

Lidholm J., Szmidt A., Gustafsson P., 1991. Duplication of the psbA gene in the chloroplast genome of two *Pinus* species. Molecular and General Genetics. 226 (3) 345-352.

Liebl U., Mockenstrum-Wilson M., Trost JT., Brune DC., Blankenship RE., and Vermaas W. Single core polypeptide in the reaction centre of the photosynthetic bacterium *Heliobacillus mobilis:* structural implications and relations to other photosystems. Proceed. Nation. Acad. Scie. USA. 90. 7124-7128

Los DA, Zorina A, Sinetova M, Kryazhov S Mironov K and Zinchenko V. 2010. Stress sensors and signal Transducers in Cyanobacteria. Sensors. 10: 2386-2415

Lundell DJ., Williams RC., Glazer AN. 1981. Molecular architecture of a light-harvesting antenna. In vitro assembly of the rod substructures of *Synechococcus* 6301 phycobilisomes. J. Biol. Chemistry. 256. 3580-3592.

Lutkenhaus, J., & Addinall, S.G., 1997. Bacterial cell division and the Z ring. Annual Review of Biochemistry. 66: 93-116.

MacColl R. 1998. Cyanobacterial Phycobilisomes. J. Structural Biol. 124. 311-334

MacColl R., Williams O., Eisele LE., Berns DS. 1994. Spectroscopic changes tor C-phycocyanin and phycoerythrin 545 produced by ferric ion. Biochim. Biophys. Acta - Bioenergetics. 1188(3). 398-404.

Madigan M; Martinko J (editors). 2005. Brock Biology of Microorganisms. Eleventh ed. Prentice Hall. I

Magnuson, A., Rova, M., Mamedov, F. and Styring, S. 1998. The role of cytochrome b559 and tyrosine D in protein against photoinhibition during in vivo photoactivation of Photystem II. Biochim. Biophys. Acta 1411, 180-191;

Mann N. H., Novac. N., Mullineaux C.W., Newman. J, Bailey. S., Robinson. C., 2000. Involvement of an FtsH homologue in the assembly of functional photosystem I in the cyanobacterium Synechocystis sp. PCC6803. FEBS Letters 479, 72-77Wentzel RG 1989, Limnology, 2nd ed. p 255-297. Saunders College Publishing Philadelphia.

Manna P., & Vermaas W. 1997. Lumenal proteins involved in the respiratory transport in the cyanobacterium *Synechocystis sp.* PCC6803. Plant Molec. Biol. 35(4):407-416

Mannan RM. and Pakrasi HB. 1993. Dark heterotrophic growth conditions result in an increase in the content of photosystem II units in the filamentous cyanobacterium *Anabaena variabilis* ATCC 29413. Plant Physiol. 103. 971-977

Manodori A & Melis A. 1985. Phycobilisome - photosystem II association in *Synechococcus* 6301 (*Cyanophyceae*). FEBS Letters. 181. 79-82

Manodori A. & Melis A. 1986. Cyanobacterial acclimation to photosystem I or Photosystem II light. Plant Physiol. 82(1): 185-189

Margulis L. 1996. Archaeal-eubacterial mergers in the origin of Eukarya: Phylogenetic classification of life. Proc. Nation. Acad. Scie USA/ 93. 1071-1076.

Marin B, Nowack ECM, Melkonian M. 2005. A plasid in the making: evidence for a second primary endosymbiosis. Protist. 156: 425—432.

Marin, B., Nowack, E. C. M. & Melkonian, M. 2005. A plastid in the making: Evidence for a second primary endosymbiosis. Protist 156, 425–432.

Martin RE., Thomas DJ., Tucker DE., Herbert SK. The effects of photo oxidative stress on photosystem I measured in vivo in Chlamydomonas. Plant Cell Environment. 20(12): 1451-1461.

Matthews BW & Fenna RE. 1980. Structure of a green bacteriochlorophyll protein. Accounts Chemic. Research. 13(9). 309-317

Mattoo AK., Hoffman-Falk H., Marder JB., & Edelman M. 1984. Regulation of protein metabolism: coupling of photosynthetic electron transport to *in vivo* degradation of the rapidly metabolised 32-kilodalton protein of the chloroplast membranes. Proc. Nation. Acad. Sci. USA. 81: 1380-1384

Mayes SR, Dubbs JM, Vass I, Hideg E, Nagy L, Barber J. 1993. Further characterisation of the psbH locus of Synechocystis sp. PCC 6803: Inactivation of psbH impairs QA to QB electron transport in photosystem II. Biochemistry 32:1454-65

Merchant S. & Dreyfuss BW. Posttranslational assembly of photosynthetic metalloproteases. Ann. Rev. Plant. Physiol. Plant. Mol. Biol. 49: 25-51

Michael, HP. and Bennett, J. 1987. Identification of the phosphorylation site of an 8.3kDa protein from photosystem II of spinach. *FEBS Lett.* **212**, 103-108.

Michel H., & Deisenhofer J. 1988. Relevance of the photosynthetic reaction centre from purple bacteria to the structure of photosystem II. Biochemistry. 27(1): 1-7

Mimuro M, Kikuchi H. Murakami A. 1999. Structure and Function of phycobilisomes. in: Concepts in Photobiology. Photosynthesis and photomorphogenesis. pp. 104-137. Kluwer Academic Publishers

Molouba F, Lorquin J, Willems A, Hoste B, Giraoud E, Dreyfus B, Gillis M, Philiippe de Lajudie, and Masson-Boivin C. 1999. Photosynthetic Bradyrhizobia from Aeschynomene spp. Are Specific to Stem-Nodulated Species and Form a Separate 16S Ribosomal DNA Restriction Fragment Length Polymorphism Group. Applied & Environmental Microbiology. 3084-3094

Mörschel E., & Schatz GH. 1987. Correlation of photosystem II complexes with exoplasmatic freeze-fracture particles of thylakoids of the cyanobacterium *Synechocystis sp.* Planta. 172(2): 145-154.

Mühlenhoff U., & Chauvat F. 1996. Gene transfer in the thermophilic cyanobacterium *Synechococcus eleongaturs*. Molecul. General Genetics. 252: 93-100.

Mullineaux C.W., and Holzwarth, A.R. 1993. Effect of photosystem II reaction centre closure on fluorescence decay kinetics in a cyanobacterium. Biochim. Biophys. Acta 1183, 345–351.

Mullineaux CW 1992. Excitation energy transfer from phycobilisomes to photosystem I in a cyanobacterium. Biochim Biophys Acta 1100: 285-292

Mullineaux CW 1994. Excitation energy transfer from phycobilisomes to photosystem I in a cyanobacterial mutant lacking Photosystem II. Biochim Biophys Acta 1184: 71-77.

Mullineaux CW 1999. The thylakoid membranes of cyanobacteria; Structure, dynamics and function. Aust. J Plant Physiol 26: 671-677

Mullineaux CW. 1992. Excitation energy transfer from phycobilisomes to photosystem I in a cyanobacterium. Biochim Biophys Acta 1100: 285-292

Mullineaux CW. 2001. Micro Review. How do cyanobacteria sense and respond to light? Molecular Microbiology. 41(5): 965-971.

Mullineaux CW. and Sarcina M., 2002. Probing the dynamics of photosynthetic membranes with fluorescence recovery after photobleaching. TRENDS Plant Scie. 7(6): 237-240.

Mullineaux CW., 2004. FRAP analysis of photosynthetic membranes. J. Experimental Botany. 55(400): 1207-1211.

Mullineaux CW., and Emlyn-Jones D. 2005. State transitions: an example of acclimation to low-light stress. J. Exper Botany vol56. No 411; pp 389-393

Mullineaux CW., Tobin MJ., & Jones GR. 1997. Mobility of photosynthetic complexes in thylakoid membranes. Nature. 390: 421-424

Munne-Bosch S, Alegre L 2002. The function of tocopherols and tocotrienols in plants. Crit Rev Plant Sci 21:31–57

Munne-Bosch S, Alegre L. 2002. The function of tocopherols and tocotrienols in plants. Crit Rev Plant Sci 21:31–57

Murakami A and Fujita Y (1991b) Regulation of photosystem stoi- chiometry in the photosynthetic system of the cyanophyte Syne- chocystis PCC 6714 in response to light-intensity. Plant Cell Physiol 32: 223–230

Murata N, Nishimura M and Takamiya A. 1966. Fluorescence of chlorophyll in photosynthetic systems. III. Emission and action spectra of fluorescence – three emission bands of chlorophyll *a* and the energy transfer between two pigment systems. Biochim Biophys Acta 126: 234–243.

Murata N. 1969. Control of excitation transfer in photosynthesis. Light- induced change of chlorophyll a fluorescence in Porphyridium cruentum. Biochim Biophys Acta 172: 242–251

Murphy RC., Gasparich GE., Bryant DA., Porter RD. 1990. Nucleotide sequence and further characterisation of the *Synechococcus sp.* strain PCC 7002 recA gene: complementation of a cyanobacterial recA mutation by the *Escherichia coli* recA gene. J. Bacteriol. 172(2): 967-976.

Murphy. DJ. 1986. Reconstitution of energy transfer and electron transfer between solubilised pigment-protein complexes from thylakoid membranes. The role of acyl lipids. Photosynth. Research. 8(3). 219-233

Mustardy L., Cunningham FX., and Gantt E. 1992. Photosynthetic membrane topography: Quantitative *in situ* localisation of photosystems I and II. Proc. Nation. Acad. Scie. USA. 89: 10021-10025.

Mutsuda, M., Michel, K.P., Zhang, X.F., Montgomery, B.L., & Golden, S.S. 2003. Biochemical properties of CikA, an unusual phytochrome-like histidine protein kinase that resets the circadian clock in *Synechococcus elongatus* PCC7942. J. Biol. Chem. 278, 19102–19110.

Myers J, Graham JR and Wang RT (1980) Light harvesting in Anacystis nidulans studied in pigment mutants. Plant Physiol 66: 1144–1149

Nakatani HS, Ke B, Dolan E and Arntzen CJ 1984. Identity of the Photosystem II reaction center polyptide. Biochim Biophys Acta 765: 347-352.

Narberhaus F., Obrist M., Furrier F., Langklotz S. 2009. Degradation of cytoplasmic substrates by FtsH, a membrane-anchored proteases with many talents. Res. Microbiol. 160:652-659.

Nield, J., Orlova, E.V., Morris, E.P., Owen, B., van Heel, M. and Barber, J. 2000. 3D map of the plant Photosystem II supercomplex obtained by cryoelectron microscopy and single particle analysis. *Nature Struc. Biol*. **7**, 44-47.

Nilsson F., Simpson DJ., Jansson C, and Anderson B. 1992. Ultrastructure and biochemical characterisation of a *Synechocystis* 6803 mutant with inactivated psbA genes. Arch. Biochem. Biophys. 295(2): 340-347.

Nishiyama Y, Allakhverdiev SI, Yamamoto H, Hayashi H, Murata N. 2004. Singlet oxygen inhibits the repair of photosystem II by suppressing the translation elongation of the D1 protein in *Synechocystis* sp. PCC 6803. Biochemistry 43:11321–11330.

Nishiyama Y, Yamamoto H, Allakhverdiev SI, Inaba M, Yokota A., & Murata N. 2001. Oxidative stress inhibits the repair of photodamage to the photosynthetic machinery. The EMBO Journal (2001) 20, 5587 - 5594

Nixon PJ., Barker M., Boehm M., deVries R., Komenda J. 2005.FtsH-mediated repair of the photosystem II complex in response to light stress. J. Experim. Botany. 56: 357-363.

Nixon P, Michoux F, Yu J, Boehm M & Komenda J. 2010. Recent advances in understanding the assembly and repair of photosystem II. Annals of Botany. 106: 1-16

Nowack EC, Melkonian M, Glöckner G. 2008. Chromatophore genome of *Paulinella* sheds light on acquisition of photosynthesis by eukaryotes. Curr. Boil. 18(6): 410-418.

Nugent JHA., Rich AM., Evans MCW. 2001. Photosynthetic water oxidation: towards a mechanism. Biochim. Biophys. Acta. -Bioenerg. 1503: 138-146

Odom, W.R. and Bricker, T.M. 1992. Interaction of Cpa-1 with the Manganese Stabilizing protein of photosystemII: Identification of domains cross-linked by 1-ethyl-3-[3-(dimethylamino)-propyl] carbimide. *Biochemistry*. **31**, 5616-5620.

Ogura T, Koichi I, Tatsuta T., Suzaki T, Karata K, Young K, Su LH, Fierke CA, Jackman JE, Raetz CRH, Coleman J,Tomoyasu T, and Matsuzawa H. 1999. Balanced biosynthesis of major membrane components through regulated degradation of the committed enzyme of lipid A biosynthesis by the AAA protease FtsH (HflB) in Escherichia coli. Molecular Microbiology. 31. 833-844.

Ogura T., Tomoyasu T, Yuki T, Morimura S, Begg KJ, Donachie WD, Mori H, Niki H., Hiraga S. 1991. Structure and function of the ftsH gene in Escherichia coli. Research in Microbiology. 142: 279-282

Ohad I, Kyle DJ, Hirschberg J. 1985. Light-dependent degradation of the QB-protein in isolated pea thylakoids. EMBO J 4:1655–1659

Ohkawa H., Pakrasi HB., Ogawa T. 2000. Two types of functionally distinct NAD(P) H dehydrogenases in *Synechocystis sp* strain PCC 6803. J. Biol. Chemistry. 275: 31630-31634.

Ohkawa H., Price GD., Badger MR., Ogawa T. 2000. Mutation of ndh genes leads to inhibition of CO2 uptake rather than -HCO3 uptake in *Synechocystis sp.* strain PCC 6803. J. Bacteriology. 182(9): 2591-2596.

Olive J., Ajlanin G., Astier C., Recouvreur M., Vernotte C. 1997. Ultrastructure and light adaptation of phycobilisome mutants of *Synechocystis* PCC 6803. Biochim. Biophys. Acta. Bionerg. 1319. 275-282

Olson JM., 1998. Chlorophyll organisation and function in green photosynthetic bacteria. Photochemistry & Photobiology. 67(1). 61-75

Onai, K., Morishita, M., Kaneko, T., Tabata, S. and Ishiura, M. 2004. Natural transformation of the thermophilic cyanobacterium *Thermosynechococcus elongatus* BP-1: a simple and efficient method for gene transfer. *Molec. Genet. Genomics.* **271**, 50-59

Ort DR., and Yocum CF. 1996. Oxygenic Photosynthesis: the light reactions. Kluwer Academic Publishers. Dordrecht.

Osmond B and Föster B. 2008. Photoinhibition: Then and Now. In: Photoprotection, Photoinhibition, Gene Regulation and Environment. Advances in Photosynthesis and Respiration. Vol.21, pp 13-19. Springer.

Osmond, C.B. 1981. Photorespiration and photoinhibition: some implications for the energetics of photosynthesis. Biochim. Biophys. Acta 639: 77–98.

Ostersetzer, O. and Adam, Z. 1997. Light-stimulated degradation of an unassembled Rieske FeS protein by a thylakoid-bound protease: the possible role of the FtsH protease. Plant Cell. 9: 957-965.

Oudot-Le Secq, M.-P., Grimwood J, Shapiro H, Armbrust V, Chris Bowler C, Green BR. 2007. Chloroplast genomes of the diatoms *Phaeodactylum tricornutum* and *Thalassiosira pseudonana*: comparison with other plastid genomes of the red lineage. Mol. Gen. Genom. 277, 427–439.

Packham, NK. 1988. Is the 9kDa thylakoid membrane photosphorylation functionally and structurally analogous to the "H" subunit of bacterial reaction centres?. *FEBS Lett.* **231**, 284-290.

Page, R.D.M. 1996. TreeView: An application to display phylogenetic trees on personal computers. *Comput. Appl. Biosci.* **12,** 357-358.

Pakrasi HB, Riethman HC, Sherman LA 1985a Membrane development in the cyanobacterium *Anacystis nidulans* during recovery from iron starvation. Plant Physiol 79:290–295

Pakrasi HB, Riethman HC, Sherman LA 1985b. Organization of pigment proteins in the photosystem II complex of the cyanobacterium Anacystis nidulans R2. Proc Natl Acad Sci USA82:6903–6907

Pakrasi HB., Vermaas WFJ. 1992. Protein engineering of photosystem II. In J. Barber ed. The photosystems: structure, function and molecular biology. Elsevier. NY. pp231-257.

Pakrasi, HB., Decienchi, P., Whitmarsh J. 1991. Site directed mutagenesis of the heme axial ligands of cytochrome-b-559 affects the stability of the photosystem II complex. *EMBO J.***10**, 1619-1627

Park YI, Sandström S, Gustafsson P, Öquist G. 1999. Expression of the isiA gene is essential for the survival of the cyanobacterium *Synechococcus sp*. PCC 7942 by protecting photosystem II from excess light under iron limitation. Mol Microbiol 32:123–129

Park YI., Anderson JM., Chow WS. 1996. Photoinactivation of functional photosystem II and D1-protein synthesis in vivo are independent of the modulation of the photosynthetic apparatus by growth irradiance. Planta. 198(2): 300-309

Park YI., Chow WS., Anderson JM. 1995. Light inactivation of functional photosystem II in leaves of peas grown in moderate light depends on photon exposure. Planta. 196(3): 401-411

Park, Y.I., Chow, W.S., Osmond, C.B. and Anderson, J.M. 1996. Electron transport to oxygen mitigates against the photoinactivation of photosystem II in vivo. Photosynth. Res. 50: 23–32.

Parkrasi, HB., Nyhus KJ, Granok H. 1990. Targeted deletion mutagenesis of the beta-subunit of cytochrome-b559 protein destabilizes the reaction centre of photosystem II. *Z. Naturfotshch* **45**, 423-429

Partensky F., Blanchot J., Vaulot D. 1999. Differential distribution and ecology of Prochlorococcus and Synechococcus in oceanic waters: a review. Bulletin de l'Institute océanographique, Monaco, No 19

Partensky F., Hess WR., Vaulot D. 1999 *Prochlorococcus*, a Marine Photosynthetic Prokaryote of Global Significance. Microbiol. Mol. Biol. Rev. vol. 63 no. 1 106-127

Passoni, S. & C. Callieri. 2001. Picocyanobacteria single forms, aggregates and microcolonies: survival strategy or species succession? *Verh. Internat. Verein. Limnol.,* 27: 1879-1883

Peschek GA., 1996. Cytochrome oxidase and the cat operon of cyanobacteria. Bioch. Biophys. Acta - Bioenerget. 1275: 27-32.

Pfündel E 1998. Estimating the contricution of Photosystem I to toal leaf chlorophyll fluorescence. Photosynth. Res. 56; 185-195.

Pohlschröder M, Hartmann E, Hand NJ, Dilks K. Haddad A. 2005. Diversity and Evolution of Protein Translocatio. Annu. Rev. Microbiol. 2005. 59:91–111

Porra RJ., Thompson WA., Kriedemann. PE., 1989. Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophyll *a* and *b* extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. Biochim. Biphys.Acta. 975. 384-394.

Prasil, O., Adir, N., and Ohad, I. 1992. Dynamics of photosystem II: Mechanism of photoinhibition and recovery processes. In The Photo- systems: Structure, Function and Molecular Biology, J. Barber, ed (Amsterdam: Elsevier Science Publishers), pp. 295–348.

Prokhorenko VI. & Holzwarth AR. 2000. Primary processes and structure of the photosystem II reaction centre: A photon echo study. J. Phys. Chem. B. 104(48): 111563-11578.

Putnam-Evans, C. Bricker, T.M. 1992. Site-directed mutagenesis of the CP-1 protein of photosystem II: Alteration of the basic pair 384, 385R to 484, 385G leads to a defect association with the O_2 evolving complex. *Biochemistry*. **31**, 11482-11488.

Quaiser, A., Ochsenreiter, T., Lanz, C., Schuster, S. C., Treusch, A. H., Eck, J., & Schleper, C. 2003. Acidobacteria form a coherent but highly diverse group within the bacterial domain: evidence from environmental genomics. Molecular microbiology, 50(2), 563-575.

Raven J.A., Evans MCW., Korb RE. The role of trace metals in photosynthetic electron transport in O2-evolving organisms. Photosynt. Research. 60: 111-150

Reith, M. 1995. Molecular biology of rhodophyte and chromophyte plastids. Annu. Rev. Plant Physiol. Plant Mol. Biol. 46, 549-575.

Reithman, H., G. Bulleijahn, K. J. Reddy, and L. A. Sherman. 1988. Regulation of cyanobacterial pigment-protein composition and organization by environmental factors. Photosynth. Res. 18:133-161.

Rep. M., & Grivell LA., 1996. The role of protein degradation in migotchondrial function and biogenesis. Current Genetics. 30(5). 367-380

Reuter W., Westermann M., Brass S., Ernst A., Böger P., Wehrmeyer W. Structure, composition, and assembly of paracrystaline phycobiliproteins in *Synechocystis sp.* strain BO8402 and of phycobilisomes in the derivative strain BO9201. J. Bacteriol. 176(3). 896-904

Rhee, K-H. 2001. Photosystem II: The solid structural era. *Ann. Rev. Biophys. Biomol. Struct.* **30,** 307-328

Rhee, K-H., Morris, E.P., Barber, J. and Kuhlbrandt, W. 1998. Three dimensional structure of the plant Photosystem II reaction centre at 8 Ångstrom resolution. *Nature*, **396**, 283-286

Richardson DL., Reed RH,, Stewart WDP. 1983. *Synechocystis PCC6803:* a eurlyhline cyanobacterium. FEMS Microbiology Letters. 18: 99-102.

Rijgersberg CP, Amesz J, Thielen A PGM and Swager JA 1979. Fluorescence emission spectra of chloroplast preparations at low temperature. Biochim Biophys Acta 545: 473-482.

Rippka, R., Deruelles, J., Waterbury, J.B., Herdman, M and Stanier, R.Y. 1979. Generic Assignments, strain histories and properties of pure cultures of cyanobacteria. *J. General Microbiol.* **111**, 1-61.

Robertson, B.R., N.N. Tezuka & M.M. Watanabe. 2001. Phylogenetic analyses of Synechococcus strains (cyanobacteria) using sequences of the 16S rDNA and part of the phy- cocyanin operon reveal multiple evolutionary lines and reflect phycobilin content. Internat. J. Syst. Evol. Microbiol., 51: 861-871.

Robinson C. & Bolhuis A. 2004. Tat-dependent protein targeting in prokaryotes and chloroplasts. Biochim. Biophysica Acta 1694: 135–147.

Rodrigues RAO., Silva-Filho MC., Cline K. 2011. FtsH2 and FtsH5: two homologous subunits use different integration mechanisms leading to the same thylakoid multimeric complex. The Plant J. 65: 600-609.

Roegner, M., Chisholm, DA., Diner, BA. 1991. Site directed mutagenensis of the psbC gene of photosystem II. Isolation and functional characterization of CP43-less photosystem II core complexes. *Biochemistry.***30**, 5387-5395.

Roger P.A., Kulasooriya S.A., 1980. in: Blue-green algae and rice. International Rice Research Institute.

Romer, S., Senger, H. and Bishop, N.I. 1995. Characterization of the carotenoidless strain of *Scenedesmus obliquus,* mutant C-6E, a living Photosystem I model. *Bot. Acta.* **108**, 80-86

Saitou, N. and Nei, M. 1987. The neighnor-joining method a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4,** 406-425.

Sakamoto W, Zaltsman A, Adam Z, Takahashi Y. 2003. Coordinated regulation and complex formation of YELLOW VARIEGATED1 and YELLOW VARIEGATED2, chloroplastic FtsH metalloproteases involved in the repair cycle of photosystem II in Arabidopsis thylakoid membranes. The Plant Cell 15: 2843–2855.

Sakamoto W. 2003. Leaf-variegated mutations and their responsisble genes in Arabidopsis thaliana. Genes Genet. Syst. 78: 1-9

Salehian O and Bruce D 1992. Distribution of excitation energy in photosynthesis: Quantification of fluorescence yields from intact cyanobacteria. J Luminescence 51: 91–98

Sambrook J., & Russel D. 2001. Molecular cloning; A Laboratory manual. 2nd volume. Cold Spring Harbor Laboratory Press, New York. USA.

Samsonoff WA. MacColl R. 2001. Biliproteins and phycobilisomes from cyanobacteria and red algae at the extremes of habitat. Archives Microbiol. 176. 400-405

Sandström S, Ivanov AG, Park YI, Öquist G, Gustafsson P. 2002. Iron stress responses in the cyanobacterium *Synechococcus sp.* PCC7942. Physiol Plant 116:255–263

Sandström S, Park YI, Öquist G, Gustafsson P. 2001. CP43', the isiA gene product, functions as an excitation energy dissipator in the cyanobacterium *Synechococcus sp*. PCC 7942. Photochem Photobiol 74:431–437

Sára M, & Sleytr UB. 2000. "S-Layer proteins". J. Bacteriol. 182 (4): 859–68.

Sarcina M., & Mullineaux CW. 2004. Mobility of the IsiA chlorophyll-binding protein in cyanobacterial thylakoid membranes. J. Biolog. Chem. 279(35): 36514-36518.

Sarcina M., Bouzovitis N., & Mullineaux CW. 2006. Mobilization of photosystem II induced by intense red light in the cyanobacterium *Synechococcus sp* PCC 7942. Plant Cell. 18. 457-464.

Sarcina M., Tobin MJ., & Mullineaux CW. 2001. Diffusion of phycobilisomes on the thylakoid membranes of the cyanobacterium *Synechococcus* 7942. J. Biolog. Chem. 276(50): 46830-46834

Satoh K 1980. F-695 emission from the purified Photosystem II chlorophyll a-protein complex. FEBS Lett 110: 53–56

Satoh K. 1970. Mechanism of photoinactivation in photosynthetic systems I. The dark reaction in photoinactivation. Plant & Cell Physiology. 11(1). 15-27

Scanlan DJ. & West NJ., 2002. Molecular ecology of the marine cyanobacterial genera *Prochlorococcus* and *Synechococcus*. FEMS Microbiology Letters Ecology. 40: 1-12

Scheller HV. 1996. In Vitro cyclic electron transport in barley thylakoids follows two independent pathways. Plant Physiol. 110(1): 187-194

Schilstra MJ., Rappaport F., Nugent JH., Barnett CJ., and Klug DR. Proton/Hydrogen transfer affects the S-state-dependent Microsecond phases of P680+ Reduction during water splitting. Biochemistry, 1998, 37(11): 3974-3981.

Schuster G, Timberg R, Ohad I. 1988. Turnover of thylakoid photosystemII proteins during photoinhibition of Chlamydo- monas reinhardtii. Eur J Biochem 177:403–410.

Sharma J, Panico M, Shipton CA, Nilsson F, Morris HR, Barber J. 1997. Primary structure characterization of the photosystem II D1 and D2 subunits. J Biol Chem 272:33158–33166.

Shen G and Vermaas WFJ 1994. Chlorophyll in a Synechocystis sp. PCC 6803 mutant without Photosystem I and Photosystem II core complexes. Evidence for peripheral antenna chlorophylls in cyanobacteria. J Biol Chem 269: 13904–1391

Sherman DM, Sherman LA 1983. Effect of iron deficiency and iron restoration on ultrastructure of Anacystis nidulans. J Bacteriol 156:393–401

Sherman DM., Troyan TA., and Sherman LA. 1994. Localisation of membrane proteins in the cyanobacterium *Synechococcus sp.* PCC7942 (radial asymmetry in the photosynthetic complexes).

Shestakov SV., & Khyen NT. 1970. Evidence for genetic transformation in blue-green alga *Anacystis nidulans.* Molecul. General Genetics. 107(4): 372-375

Shikanai T., Endo T., Hashimoto T., Yamada Y., Asada K., Yokota A. Directed disruption of the tobaco ndhB gene impairs cyclic electron flow around photosystem I. Proc. Nat. Acad. Scie. USA. 95(16): 9705-9709

Shotland, Y., Koby, S., Teff, D., Mansur, N., Oren, D.A., Tetematsu, K., Tomoyasu, T., Kessel, M., Bukau, B., Ogura, T., and Oppenheim, A.B. (1997) Proteolysis of the phage l CII regulator y protein by FtsH (HflB) of Escher i- chia coli. Mol Microbiol 24: 1303–1310.

Sidler, W.A. 1994. In The Molecular Biology of Cyanobacteria, D. Bryant, ed (Dordrecht, The Netherlands: Kluwer Academic Publishers), pp. 139–216.

Silva P, Thompson E, Bailey S, Kruse O, Mullineaux CW, Robinson C, Mann NH, Nixon PJ. 2003. FtsH is involved in the early stages of repair of photosystem II in Synechocystis sp PCC 6803. The Plant Cell 15: 2152–2164

Singh AK, Sherman LA. 2006. Iron-independent dynamics of IsiA production during the transition to stationary phase in the cyanobacterium *Synechocystis sp*. PCC 6803. FEMS Microbiol Lett 256:159–164

Sokolenko, A., Pojidaeva, E., Zinchenko, V., Panichkin, V., Glaser, V.M., Herrmann, R.G., and Shestakov, S.V. 2002. The gene complement for proteolysis in the cyanobacterium *Synechocystis* sp. PCC 6803 and *Arabidopsis* thaliana chloroplasts. Curr. Genet. 41, 291–310.

Sonoike K & Terashima I. 1994. Mechanism of photosystem-I photoinhibition in leaves of *Cucumis sativus* L. Planta. 194; 287-293

Sonoike K. 1996. Photoinhibition of Photosystem I: its physiological significance in the chilling sensitivity of plants. Plant & Cell Physiology. 37:(3) 239-247

Sopory SK, Greenberg BM, Mehta RA, Edelman M, Mattoo AK (1990) Free radical scavengers inhibit light-dependent degrada- tion of the 32 kDa photosystem II reaction center protein. Z Naturforsch 45c:412–417

Steglich C, Behrenfeld M, Koblizek M, Claustre H, Penno S, Prasil O, Partensky F, Hess WR. 2001. Nitrogen deprivation strongly affects Photosystem II but not phycoerythrin level in the divinyl-chlorophyll b-containing cyanobacterium Prochlorococcus marinus. Biochim Biophys Acta - Bioenergetics. Vol 1503, issue 3. pp 341-349

Steglich G, Neupert W, Langer T. 1999. Prohibitins regulate membrane protein degradation by the m-AAA protease in mito- chondria. Molecular & Cellular Biology. 19: 3435–3442.

Stemler A. & Murphy J., 1984. Inhibition of HCO3- Binding to photosystem II by Atrazine at low-affinity herbicide binding site. Plant Physiol. 76, 179-182.

Steunou AS, Devaki B, Bateson MM, Melendrez MC, Ward DM, Brecht E, Peters JW, Kühl M, Grossman AR. 2006. In situ analysis of nitrogen fixation and metabolic switching in unicellular thermophilic cyanobacteria inhabiting hot spring microbial mats. PNAS 103 (7) 2398-2403;

Stockner, J., C. Callieri & G. Cronberg. 2000. Picoplankton and other non-bloom forming cyanobacteria in lakes. In: B. Whitton & M. Potts (Eds), *Ecology of Cyanobacteria: Their Diversity in Time and Space.* Kluwer Academic Publishers: 195-238.

Summer EJ , Mori H, Settles AM, and Cline K. 2000. The Thylakoid ΔpH-dependent Pathway Machinery Facilitates RR-independent N-Tail Protein Integration. J. Biologic. Chemis. 275(31): 23483–23490.

Swaffield, J.C. & Purugganan, M.N. 1997. The evolution of the conserved ATPase domain (CAD): reconstructing the history of and ancient protein module. *J. Molec. Evol.* **45**, 549-563

Takahashi S & Murata N. 2008. How do environmental stresses accelerate photoinhibition. Trends in Plant Science 13(4). 178-182

Takahashi Y, Matsumoto H, Goldschmidt Clermont M, Rochaix JD. 1994. Directed disruption of the Chlamydomonas chloroplast psbK gene destabilizes the photosystem II reaction-center complex. Plant Mol. Biol. 24: 779-88

Tanifuji G, Onodera NT, Wheeler TJ, Dlutek M, Donaher N, Archibald JM. 2011. Complete nucleomorph genome sequence of the nonphotosynthetic alga Cryptomonas paramecium reveals a core nucleomorph gene set. Genome Biol Evol. 3:44-54

Tepperman JM, Zhu T, Chang H-S. Wang X, Quail PH. Multiple transcription-factor genes are early targets of phytochrome A signaling. Proceedings National Academy Science. 98(16): 9437-9442.

Thiel T., & Poo H. 1989. Transformation of a filamentous cyanobacterium by electroporation. J. Bacteriol. 171(10): 5743-5746.

Thompson , J.D., Higgins, D.G. and Gibson, T.J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22,** 4673-4680.

Thompson WF & White MJ. 1991. Physiological and molecular studies of light-regulated nuclear genes in higher plants. Annual Review Plant Physiol. Plant Molecul. Biol. 42. 423-466.

Tian L., van Stokkum IHM., Koehorst RBM., Jongerius A., Kirilovsky D., & vanAmerogen H. 2011. Site, Rate and mechanism of photoprotective quenching in cyanobacteria. J. American Chemical Society. 133: 18304-18311.

Tobin E.M & Silverthorne. 1985. Light regulation of gene expression in higher plants. Annual Review Plant Physiol. 36. 369-393

Tomitani A., Knoll AH., Cavanaugh CM., Ohno T. 2006. The evolutionary diversification of cyanobacteria: molecular-phylogenetic and paleontological perspectives. PNAS. 103(14): 5442- 5447

Tommos C., & Babcock GT. 2000. Proton and hydrogen currents in photosynthetic wate oxidation. Biochim. Biophys. Acta - Bioenerg. 1458: 199-219.

Tomoyasu, T., Gamer, J., Bukau, B., Kanemori, M., Mori, H., Rutman, A.J., Oppenheim, A.B., Yura, T., Yamanaka, K., Niki, H., Higara, S. and Ogura, T. 1995. *Escherichia coli* FtsH is a membrane-bound, ATP-dependent protease which degrades the heat shock transcription factor σ^{32}. *EMBO J.* **14,** 2551-2560.

Tomoyasu, T., Yuki, T., Morimura, S., Mori, H., Yamanaka, K., Hiki, H., Higara, S. and Ogura, T. 1993. The *Escherichia coli* FtsH protein is a prokaryotic member of a protein family of putative ATPases involved in membrane functions, cell cycle control, and gene expression. *J. Bacteriol.* **175,** 1344-1351

Tracewell C., & Brudvig G. 2001. Species dependent differences in the secondary electron-donation reactions of photosystem II. *PS2001* **3** ,CSIRO publishing.

Trebst A, Depka B, Hollander-Czytko H. 2002. A specific role for tocopherol and of chemical singlet oxygen quenchers in the maintenance of photosystem II structure and function in Chlamydomonas reinhardtii. FEBS Lett 516:156–160.

Tsinoremas NF., Schaefer MR, and Golden S. 1994, Blue and Red Light Reversibly Control psbA Expression in the Cyanobacterium *Synechococcus* sp. Strain PCC 7942. J. Biological Chemistry. 269(23): 16143-16147.

van Rensen, J.J.S., Xu, C.H. and Govindjee (1999) Role of bicarbonate in photosystem II, the water plastoquinone oxido-reductase of plant photosynthesis. *Physiol*. *Plant*. **105**, 585-592.

van Thor JJ., Mullineaux CW., Matthijs HCP., Hellingwerf KJ. 1998. Light harvesting and state transitions in cyanobacteria. Botanica Acta. 111. 430-443.

Vass I and Cser K. 2009. Janus-faced charge recombinations in photosystem II photoinhibition. Trends in Plant Science. 14: 200-205.

Vermaas WFJ, Ikeuchi M, Inoue Y. 1988. Protein-composition of the photosystem II core complex in genetically engineered mutants of the cyanobacterium Synechocystis sp PCC-6803. Photosynth. Res. 17:97-113

Vermaas WFJ, Williams JGK, Arntzen CJ. 1987. Sequencing and modification of psbB, the gene encoding the CP-47 protein of photosystem II, in the cyanobacterium Synechocystis 6803. Plant Mol. Biol. 8:317-326

Vermaas, WFI., Williams JGK, Rutherford AW, Mathis P, Arntzen CJ. 1986. Genetically engineered mutant of the cyanobacterium Synechocystis 6803 lacks the photosystem II chlorophyll-binding protein CP-47. Proc. Natl. Acad.Sci. USA. 83: 9474-77.

Vernotte C, Astier C and Olive J 1990. State 1–state 2 adapta- tion in the cyanobacteria Synechocystis PCC 6714 wild type and Synechocystis PCC 6803 wild and phycocyanin-less mutant. Photosynth Res 26: 203–212

Vinnemeier J, Hagemann M. 1999. Identification of salt-regulated genes in the genome of the cyanobacterium *Synechocystis sp*. strain PCC 6803 by subtractive RNA hybridization. Arch Microbiol 172:377–386

Vörös, L., C. Callieri, K.V. Balogh & R. Bertoni. 1998. Freshwater picocyanobacteria along trophic gradient and light quality range. Hydrobiologia, 369/370: 117-125.

Wagner D., Przybyla D., denCamp R., Kim C., Landgraf F., Lee KP., Würsch M., Laloi C., Nater M., Hideg E., Apel K. 2004. The Genetic Basis of Singlet Oxygen–Induced Stress Responses of Arabidopsis thaliana Science. 306: 1183-118

Wagner, G. M. 1997. "Azolla: a review of its biology and utilization," *Botanical Review*, vol. 63, no. 1, pp. 1–26.

Wang RF., O'Hara EB., Aldea M., Bargmann CI., Gromley H., and Kushner SR. 1998. *Escherichia coli mrsC* is an allele of *hflB*, encoding a membrane-associated ATPase and protease that is required for mRNA decay. J Bacteriol 180: 1929–1939.

Wendler J., W., Scheer J H. Hoezwarth AR. 1986. Energy transfer in trimeric c-phycocyanin studied by picosecond fluorescence kinetics. Photochem. Photobiol. 44. 79-85.

Wentzel RG 1989, Limnology, 2nd ed. p 255-297. Saunders College Publishing Philadelphia.

Westerman M., Ernst A., Brass S, Boger P., Wehrmeyer W 1994. Ultrastracture of cell wall and photosynthetic apparatus of the phycobilisome-less *Synechocystis* sp strain BO 8402 and phycobilisome-containing derivative strain BO 9201. Archives of Microbiology 162: 222-232

Wettern M. 1986. Localization of 3200 dalton chloroplast protein pools in thylakoids: Significance in atrazine binding. Plant Science. 43, 173-177

Williams JS., Eckdahl TT., Anderson JN. 1988. Bent DNA functions as a replication enhancer in *Saccharomyces cereviseae.* Molecu. Cellular Biol. 8(7): 2763-2769.

Williams WP and Allen JF 1987. State 1 / State 2 changes in higher plants and algae. Photosynthesis Res. 13: 19-45

Wilmotte A., & Herdman M., 2001. Phylogenetic relationships among the cyanobacteria based of 16S rRNA sequences. ed. Garrity GM. : Bergey's manual of systematic bacteriology. Vol 1: The Archaea and the deeply branching and phototrophic bacteria. pp: 487-493. Publisher Williams & Wilkins.

Woese CR. 1987. Bacterial Evolution. Microbiological Reviews. 51(2). 221-271

Woese CR. 1998. The universal ancestor. Proc. Natl. Acad. Sci. USA. 95: 6854-6859

Woese CR. 2004. A new biology for a new century. Microbiol. Molecul. Biol. Reviews. 68(2). 173-186

Woese CR., & Fox GE. 1977. The concept of cellular evolution. J. Mol. Evol. 10, 1-6

Woese CR., 2000. Interpreting the universal phylogenetic tree. Proc. Nat. Acad. Scie. 97 (15). 8392-8396

Wollman, F.A., Minai, L., and Nechushtai, R. 1999. The biogenesis and assembly of photosynthetic proteins in thylakoid membranes. Biochim. Biophys. Acta 1411, 21–85.

Wong, F. Y. K., E. Stackebrandt, J. K. Ladha, D. E. Fleischman, A. R. Date, and J. A. Fuerst. 1994. Phylogenetic analysis of Bradyrhizobium japonicum and photosynthetic stem-nodulating bacteria from Aeschynomene species

Woodcock DM., Crowther PJ., Doherty J., Jefferson S., DeCruz E., Noyer-Weidner, Smith SS., Michael MZ., Graham MW. 1989. Quantitative evaluation of *Escherichia coli* host strains for tolerance to cytosine methylation in plasmid and phage recombinants. Nucleic Acid Research. 17(9): 3469-3478.

Wu QG, Vermaas WFJ. 1995. Light-dependent chlorophyll a biosynthesis upon chlL deletion in wild-type and photosystem I-less strains of the cya- nobacterium *Synechocystis* sp PCC 6803. Plant Molecular Biology 29: 933 – 945.

Wyman, M., R. P. F. Gregory, and N. G. Carr. 1985. Novel role for phycoerythrin in a marine cyanobacterium, Synechococcus strain DC2. Science 230:818-820. .

Xiong J. 2002. Complex evolution of photosynthesis. Ann. Rev. Plant Biol. 53. 503-521

Xiong J., Inoue K., Bauer CE. 1998. Tracking molecular evolution of photosynthesis by characterisation of a major photosynthesis gene cluster from *Heliobacillus mobilis*. Proceed. Nation. Acad. Scie. USA. 95(25). 14851-14856

Yamanaka G., Glazer AN., Williams RC., Cyanobacterial phycobilisomes. Characterisation of the phycobilisomes of *Synechococcus sp* 6301. J. Biolog. Chem. 253. 8303-8310.

Yamanaka, G., and A. N. Glazer. 1980. Dynamic aspects of phycobilisome structure. Phycobilisome turnover during nitrogen starvation in Synechococcus sp. Arch. Microbiol. 124:39- 47.

Yeh, K.C.; Wu, S.H.; Murphy, J.T.; Lagarias, J.C. 1997. A cyanobacterial phytochrome two-component light sensory system. *Science. 277,* 1505-1508.

Yeh, S. W., L. J. Ong, and A. N. Glazer. 1986. Role of phycoerythrin in marine picoplankton Synechococcus spp. Sci- ence 234:1422-1423.

Yoon, H. S., Hackett JD., Ciniglia C., Pinto G., Bhattacharya D. 2004. A molecular timeline for the origin of photosynthetic eukaryotes. Molecular Biology and Evolution 21, 809–818

Yoon, H. S., Reyes-Prieto A, Melkonian M, Bhattacharya D. 2006. Minimal plastid genome evolution in the Paulinella endosymbiont. Current Biology 16, R670–R672.

Yu F., Park S., and Rodermel SR. 2004. The Arabidopsis FtsH metalloprotease gene familly: intechangeability of subunits in chloroplast oligomeric complexes. The Plant J. 37: 864-876.

Yu F., Park S., and Rodermel SR. 2005. Functional Redundancy of AtFtsH metalloproteases in thylakoid membrane complexes. Plant Physiology, 138: 1957-1966.

Yu L., Zhao J., Muhlenhoff U., Bryant DA., and Golbeck JK. 1993. PsaE is required fro in vivo cyclic electron flow around Photosystem I in the cyanobacterium *Synechococcus sp* PCC 7002. Plant Physiology. 103(1): 171-180

Yu MH., Glazer AN., Williams RC. 1981. Cyanobacterial phycobilisomes. Phycocyanin assembly in the rod substructures of *Anabaena variablis* phycobilisomes. J. Biolog. Chem. 256. 13130-13136.

Zak E, Norling B, Maitra R, Huang F, Andersson B, Pakrasi HB. 2001. The initial steps of biogenesis of cyanobacterial photosystems occur in plasma membranes. Proceedings of the National Academy of Sciences, USA 98: 13443–13448.

Zaltsman, A., Ori, N. and Adam, Z. 2005. Two types of FtsH protease subunits are required for chloroplast biogenesis and Photosystem II repair in Arabidopsis. Plant Cell, 17, 2782–2790.

Zhang F., Lee GM, Jacobson K., 1993. Protein lateral mobility as a reflection of membrane microstructure. BioEssays 15. 579-588

Zhang L, Wei Q, Wu WJ, . 2009. Activation of the heterotrimeric G protein alpha-subunit GPA1 suppresses the FtsH-mediated inhibition of chloroplast development in Arabidopsis. The Plant Journal 58: 1041 – 1053.

Zhang ZH, Mayes SR, Vass I, Nagy L, Barber J. 1993. Characterisation of the psbK locus of Synechocystis sp. PCC 6803 in terms of photosystem II function. Photosynth. Res. 38:369-77

Zouni, A. Jordan, R., Schlodder, E., Fromme, P. and Witt, H.T. 2000. First Photosystem II crystals capable of water oxidation. *Biochim. Biophys. Acta.* **1457**, 103-105.

Zouni, A., Witt, H.T., Kern, J., Fromme, P., Krauss, N., Saenger, W. and Orth, P. 2001. Crystal structure of photosystem II from *Synechococcus elongatus* at 3.8 Å resolution. *Nature*. **409**, 739-743.